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Original Article

Molecular Detection of Herpesvirus, Adenovirus, and Circovirus and Their Associated Histopathological Lesions in the Pigeons of Mashhad, Iran

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ABSTRACT

Pigeon racing is a popular sport, where trained pigeons compete against each other in flying competitions. Furthermore, pigeons serve as laboratory specimens for scientific research and experiments. Additionally, many people keep pigeons as companion animals, enjoying their company and unique characteristics. The pigeon holds a significant cultural and religious significance, particularly in Islamic countries. Pigeons are susceptible to a range of viral pathogens that can impact their health and performance. This study focuses on three crucial viruses affecting pigeons: Pigeon Adenovirus, Pigeon Circovirus, and Pigeon Herpes virus. To detect these viruses in pigeons, the researchers utilized a method called Polymerase Chain Reaction (PCR). They collected liver samples from deceased pigeons referred to the Veterinary Hospital of Ferdowsi University in Mashhad, Iran. The researchers detected DNA from the samples and prepared histopathological slides following specific protocols. This study confirmed the presence of adenovirus in 15.5%, circovirus in 100%, and herpes virus in 22.5% of the studied pigeons. AdHistopathological examination was conducted on 43% of the samples. Although only one sample (3.3%) exhibited typical inclusion bodies, nearly all the samples showed varying degrees of pathological changes, including congestion, hemorrhage, and necrosis. The present study is among the few investigations into 3 important viruses in pigeons and it is necessary to pay special attention to its results and carry out additional works. Detecting 100% of the livers of sick pigeons as infected with circovirus can also be a significant result, because other manuscripts have not reported such severe contamination.

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1. Introduction

The poultry industry remains one of the most cost-effective and efficient source of animal protein that can be produced quickly, but it is still unable to meet the increasing demand for animal protein ,due to the growing potential demand (1). Although commercial broiler and layer farms provide the required protein, people are not interested in chicken meat and prefer other safe meat options. Despite this fact, poultry remains the most consumed livestock commodity in the world, especially in developing and emerging markets where production prospects have been relatively limited (2). Chickens are able to survive and reach market weight more quickly than other livestock and convert feed to meat more efficiently than larger animals, making poultry production more feasible and affordable than beef and pork for farmers in developing countries and emerging markets. The demand for poultry products is expected to continue to grow in the coming decades ,due to the growing population and urbanization, and rising income (3). Rural poultry provides high-quality protein from meat and eggs, essential vitamins, and minerals that are needed for the wellbeing of millions of undernourished people, especially pregnant women and children who often live in poverty. The chicken industry is committed to reducing the environmental impact ,resulting from the use of natural resources and byproducts of poultry production that contribute to climate change (4).

In parallel, Pigeon farming has emerged as a profitable and sustainable business, particularly in low-income regions, improving their economic status. It is a great source of extra income, especially in poor countries where family labor can be utilized for raising pigeons. Pigeon feeding fees are generally low, making it a cost-effective option for farmers. Pigeon farming can be done for profit, meat, and eggs, and is a complete guide for beginners. Pigeon meat is in highly valued demand because it is tasty and nutritious. Starting a pigeon farm requires low investment and can be done at home, making it a good option for those looking for a new business (5). Pigeons are also kept as ornamental birds, for sports, laboratory specimens, and companion animals. Pigeon racing is considered a popular sport, attracting competitors around the world with prizes of up to millions of US dollars (6).

Avian adenoviruses are classified into three genera: *Aviadenovirus*, *Siadenovirus*, and *Atadenovirus*. The aviadenoviruses that affect fowl are further divided into five species (A-E) and 11 serotypes. Additional species are known to affect turkeys, geese, ducks, and wild birds. Many of the aviadenoviral infections are subclinical, which means they do not produce any visible symptoms of diseaseand may only cause disease when birds have other concurrent infections (7). Commercially important avian adenoviruses belong to the *Aviadenovirus*, *Atadenovirus*, and *Siadenovirus* genera of the Adenoviridae. Currently, 12 serotypes are known to belong to the *Aviadenovirus* genus. The classification of avian adenoviruses is based on phylogeny, genome organization, and the lack of significant cross-immunity between different serotypes (8).

The pigeon circovirus (PiCV), belonging to the *Circovirus* genus and the Circoviridae family, stands out as one of the most notable infectious agents discovered in pigeons (9). The infection caused by circovirus in pigeons was first documented nearly three decades ago in Canada, the USA, and Australia (10, 11). The primary mode of transmission for the virus is horizontal, primarily occurring through the ingestion or inhalation of fecal material and feather dust contaminated with the virus (12). The elevated prevalence of PiCV can be attributed to the characteristics of pigeon breeding and rearing systems. Activities such as bird racing, pigeon exhibitions, and the presence of "one loft race" breeding facilities can facilitate the rapid dissemination of PiCV infections within pigeon populations. Additionally, these situations may contribute to the emergence of recombinant variants of the virus, as observed in other avian circoviruses infecting parrots (13). PiCV is known to be an immunosuppressor virus, which causes lymphoid depletion. Also, it can be remain hidden in tissues and later emerge upon the weakening of immune system (14).

Columbid herpes virus-1, CoHV-1, also referred to as pigeon herpesvirus, falcon herpesvirus, and stringid herpesvirus, was initially identified in 1945 in domestic pigeon (*Columba livia*) lofts located in the United States (15). It belongs to the family of *Herpesviridae* and is specifically classified under the genus *Herpesvirus*. When introduced into a population of pigeons that have not been previously exposed to or are immunosuppressed, CoHV-1 causes a severe and widespread disease with a high mortality rate (16, 17). Birds that manage to survive the infection remain carriers of the virus throughout their lives, shedding it primarily during the breeding season. Although young birds receive some protection from

maternal antibodies, they can still become latently infected (18). The initial viral infection typically occurs in the mucous membranes of the conjunctiva, respiratory system, and digestive tract, followed by the spread of the virus through the bloodstream. Common clinical manifestations include conjunctivitis and the presence of fibrinonecrotic exudate in the nasopharynx (16).

There are several ways to detect the mentioned viruses in pigeons. PCR is a widely used molecular biology technique for the detection of viral DNA. It allows for the specific amplification of viral genetic material, making it highly sensitive and specific. Virus isolation involves attempting to grow the virus in cell culture. It's a traditional but time-consuming technique. Histopathology involves examining tissues under a microscope for characteristic lesions caused by the viruses. In this study, we aimed to detect herpesviruses, adenoviruses, and circoviruses in Mashhad pigeons to estimate the distribution of these viruses. Furthermore, histopathological study was conducted to evaluate the associated hepatic lesions in the sampled birds.

2. Materials and Methods

2.1. Sampling

In this study, samples were taken from the livers of suspected cases of adenovirus, circovirus, and herpesvirus infections in racing pigeons. The samples were collected through referrals to the hospital and specialized veterinary polyclinic at the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad. The sampled birds were usually sick and exhibited general symptoms of the diseases. A few of the samples were obtained immediately after euthanizing the birds. Other samples were collected from carcasses that were less than 12 hours postmortem, based on the owner's statement regarding the history of the disease.In addition to liver sampling for virus detection, histopathological examination was also conducted on a portion of the liver samples. The carcasses suspected of carrying the mentioned viruses belonged to different areas in the city of Mashhad, and two samples were referred from the city of Quchan.

Seventy-one liver samples were collected from the carcass of pigeons. All cases exhibited general respiratory signs or diarrhea. Sampling for multiplex PCR were performed by sterile necropsy. For histopathological examinations, tissue samples were taken from liver and placed in 10% buffered formalin solutions.

2.2. Multiplex PCR

Primers used in our study were based upon the Freick et al. study in 2008 (19). They are presented in Table 1. The utilization of Cytochrome B, a segment of the genomic DNA of pigeons was chosen to ensure the absence of PCR restrictors.

2.3. Histopathology

When opening the carcass using sterile techniques, samples were taken using a sterile size 20 scalpel blade, with a maximum thickness of 1 centimeter. After tissue cutting, the tissue samples were transferred to wide-mouthed, lidded plastic sample containers containing 10% buffered formalin solution for fixation. It should be noted that the volume of formalin solution was at least 10-15 times the volume of tissue samples, and after 24 hours, the formalin solution was replaced. After fixation and transfer to the Pathology Laboratory of the Faculty of Veterinary Medicine in Mashhad, the samples were routinely processed through various stages of tissue processing, including dehydration, clearing, and infiltration. They were then paraffin-embedded, sectioned, and stained with hematoxylin and eosin.

3. Results

3.1. Multiplex PCR

Out of a total of 71 DNA samples tested in this study, adenovirus infection was confirmed in 11 (15.5%) cases, circovirus was detected in all 71 (100%) cases, and herpesvirus was identified in 16 (22.5%) cases using multiplex PCR testing.

From another perspective, among the 71 tested samples, circovirus infection was present in 100% of cases. Furthermore, 4.2% of cases were accompanied by adenovirus alone, 11.4% were accompanied by herpesvirus alone, and 11.4% were co-infected with adenovirus and herpesvirus (Table 2, Figure 1).

3.2. Histopathology

In the histopathological examination of the studied pigeon livers, several microscopic lesions were observed, including severe hepatocellular degeneration and necrosis, hyperemia and dilation of sinusoids, hemorrhage and infiltration of inflammatory cells. Another histopathological finding was caseous necrosis surrounded by macrophages, giant cells, lymphocytes and mild fibrous connective tissue formation.

Intranuclear viral inclusion bodies were only observed in the hepatocytes of one of the studied pigeons.

Table 1. Primers.

Virus [a]	Gene	Primer [b]	Sequence (5'-3')	Size of PCR product (bp)
PiHV	Polymerase	PiHV-s PiHV-as	gggacgctctgattaaggaat cttggtgatcagcagcagcttg	**242**
FAdV	Hexon	Hex-s Hex-as	caggcccaaytacatcgg gtgatgacgsgacatcat	**181**
PiCV	Capsid	PiCV2-s PiCV2-as	ttgaaaggttttcagcctggc aggagacgaaggacacgcctc	**325**
-----	Cytochrome B	cytB-s cytB-as	ccatccaacatctcagcatgatgaaa gcccctcagaatgatatttgtcctca	**359**

[a.] PiHV: pigeon herpesvirus; FAdV: fowl adenovirus; PiCV: pigeon circovirus.
[b.] The orientation is sense (-s) or antisense (-as).

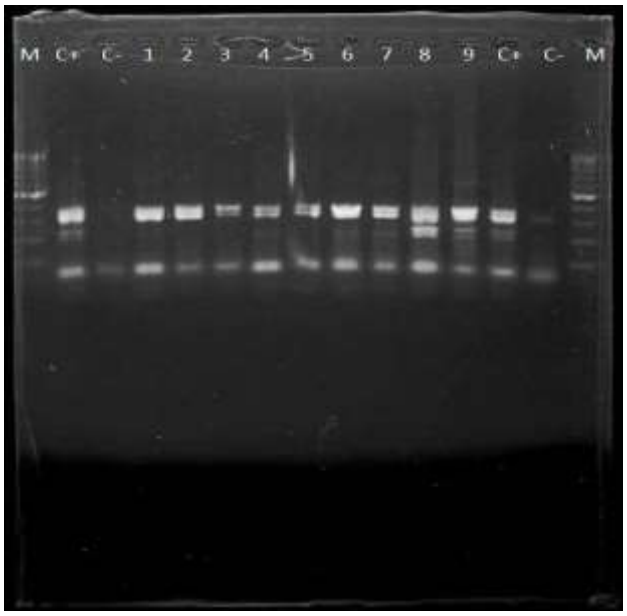

Figure 1. PCR gel. ***M*** marker, ***C+*** positive control, ***C-*** negative control. Samples are placed 1-9.

Table 2. Virus detection in samples.

Virus	All samples (71)
Circovirus	71 (100%)
Adenovirus Only	3 (4.2%)
Herpesvirus Only	8 (11.4%)
Adenovirus + Herpesvirus	8 (11.4%)

They exhibited features characteristic of Adenoviral inclusions and appeared as large basophilic bodies within the nucleus of affected hepatocytes. Within these cells, evidence of cellular injury was seen in the form of vacuolated cytoplasmic spaces (Figure 2 A-D).

4. Discussion

This research confirmed the presence of adenovirus in 15.5%, circovirus in 100%, and herpesvirus in 22.5% of the tested pigeons in Mashhad. Additionally, a histopathological sample was taken from the liver of 43% of the tested population, and an inclusion body was observed in one case (3.3%). In the study of Teske et al. (2017), a novel adenovirus (PiAdV-2), along with its two variants were discovered. The method of detection was electron microscopy of fecal samples from a YPDS (Young pigeon disease syndrome) outbreak.

Their study showed approximately 13% of adults and 20% of young pigeons harbored PiAdV-2 (20).

Rahimi Sardo et al. (2023) collected healthy and sick pigeon fecal samples, 60 of each. They used PiAdV-1 primers for detection. Only six samples were positive out of the total of 120 fecal samples. Overall, 5.00% and 3.33% of sick and healthy pigeons tested positive (21). Raue et al. (2005) in Germany were unable to prove the presence of adenovirus in any of the 45 tested pigeons using PCR (22). However, they successfully demonstrate the presence of circovirus using PCR in 45 out of 45 (100%) bursa of Fabricius samples, and in the blood of 2 out of 9 pigeons (22%).

Freick et al. in Germany (2008) stated that since all three viruses, adenovirus, circovirus, and herpesvirus, produce inclusion bodies in the liver, the liver is a suitable tissue for PCR. Sampling for their study was conducted on 45 diseased pigeons and 6 healthy pigeons.

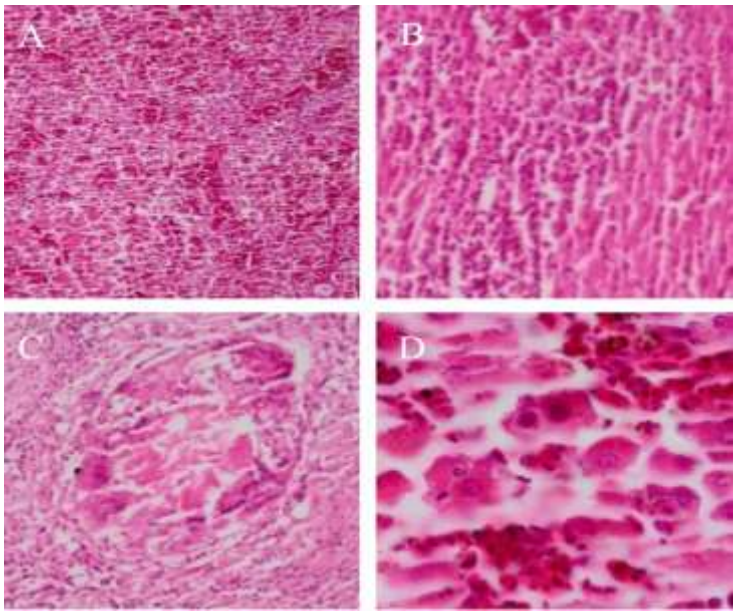

Figure 2. Histopathological figures (H&E staining method). ***A.*** Hyperemia and dilation of liver sinusoids (×20), ***B.*** Focal infiltration of inflammatory cells in the liver (×40), ***C.*** Caseous necrosis surrounded by giant cells, inflammatory cells and mild fibroplasia (×40), ***D.*** Basophilic intranuclear inclusion bodies (arrows) (×100).

The percentage of pigeons tested positive for adenovirus was reported as 0% (19). The failure to detect the virus could be due to the absence or incomplete sampling in these reports. In this study, the percentage of pigeon samples tested positive for Circovirus was reported as 88.9%.

Weissenbock and Fuchs in 1995, using liver histopathology on 226 pigeons to detect herpesvirus and adenovirus, observed inclusion bodies in 4% of them, among which 5 pigeons (2.2%) were diagnosed with adenovirus (23). Their findings align with those of this study and are considered reliable due to the large number of samples.

Catroxo et al. in 2011, in Brazil, conducted a search for viruses in the feces of 57 pigeons in Sao Paulo city using electron microscopy. Among these samples, 50 were adult pigeons and 7 were immature pigeons. The viruses identified in this study included paramyxovirus, adenovirus, and coronavirus. Out of the 57 tested samples, 2 samples (3.5%) were reported positive for adenovirus (24).

The low percentage reported by Weissenbock and Fuchs seems to be related to the method of virus detection. The presence of the virus in pigeons and its association with pathological lesions is not always consistent, and for various reasons, it is possible that the virus may not be identified through histopathology of a particular organ. Additionally, the percentage of herpesvirus infection was also low in this study. As for the findings by Catroxo et al. sampling from feces may not be suitable for adenovirus detection, and liver tissue is more suitable for this purpose. Nevertheless, it seems that reaching definitive conclusions in this regard requires more detailed and extensive research.In 2011, Ledwon et al. conducted a study in Dubai, where they collected fecal samples from 139 pigeons and tested them for the presence of circovirus and adenovirus using PCR.

They also performed the same test on the liver samples of 18 pigeons. The PCR results from the 139 tested fecal samples showed zero cases (0%) positive for adenovirus, while the test results for the 18 pigeon livers showed 8 positive cases (44%). The PCR test results showed that out of the 139 fecal samples tested, 30 samples (21%) were positive for Circovirus. Additionally, when examining 18 pigeon liver samples from a specific area of the city, circovirus was confirmed in 16 cases (89%). The results of this study indicate that fecal samples may not be reliable for adenovirus detectionIn the circovirus detection, the results of this study show a high correlation with the findings of Raue et al., Ledwon et al., and Freick et al. The high prevalence of the virus may be due to the sampling method in these studies, especially the high percentage of sick pigeons in the samples. The higher results obtained in this study compared to the three mentioned cases may be attributed to improper pigeon breeding practices in Iran and the failure of pigeon breeders to comply with health and biosafety regulations. Furthermore, the comparison of the two results obtained by Ledwon et al. confirms the high sensitivity of liver tissue in identifying Circovirus using PCR testing.

From another perspective, the proximity of the sampling location in Dubai, United Arab Emirates, to Iran and the high prevalence of Circovirus in that city further support the results of this study. Although the purchase and sale of these animals may be under the supervision of a veterinarian, as mentioned, Circovirus can be hidden, and birds can act as carriers without any clinical signs.

In 2014, Stenzel et al. conducted a PCR test for Circovirus on 324 pigeons in Poland, with 64% of urban pigeons and 44.7% of wild pigeons testing positive (25). In 2012, Cságola et al. identified PiCV in 57% of 116 tested pigeons in Hungary, with 53% of them showing no

significant symptoms (26). In 2002, Hattermann et al. confirmed the presence of PiCV in 17 out of 53 pigeons (32%) using PCR on blood samples in Germany, including both sick and healthy pigeons (27).

The results of Hattermann et al. are similar to those of Raue et al. In both cases, blood samples were used, and the reported infection rate was less than 35%, whereas Raue et al. had previously demonstrated an infection rate of over 80%. Therefore, it can be concluded that the use of blood for diagnosing pigeon infection with Circovirus, while being a non-invasive, rapid, and simple method, is also associated with low sensitivity.

In 2013, Woźniakowski et al., conducted a study in Poland involving a total of 88 birds, including pigeons, hunting birds, and non-hunting birds that had contact with pigeons. They identified 18 birds (20.4%) with CoHV-1 (28). Out of the 11 pigeons in this group, 6 (54.5%) were found to be positive for CoHV-1. The sampling in this study was performed on the brain tissue of pigeons.

Considering the site of sampling, brain tissue, and the reports by Tantawi et al. (1979) from Iraq (29), as well as Shalaby et al. (1985) from Saudi Arabia (30), indicating the tendency of herpesviruses isolated from these two countries towards neural tissue, and the proximity of these countries to Iran, and also considering that the sampling was only conducted on wild and free pigeons, the high reported percentage by Woźniakowski et al. is justified. Additionally, 11 samples of this study are relatively small for reporting and referencing the prevalence and serves mainly to demonstrate the significant presence of the virus.

Out of the 30 samples taken for histopathological examination in this study, all of them showed congestion (100%). Twenty-one samples (70%) exhibited multifocal necrosis, but only one sample (3.3%) displayed intranuclear inclusion bodies. The PCR result of this sample confirmed the presence of both circovirus and herpesvirus in the liver.

Huang et al., (2017) studied the circovirus infection in disqualified racing pigeons from Taiwan using histopathology and PCR of different tissues. Severe histopathological abnormalities, characterized by distinct structures called inclusion bodies, appearing as basophilic globules, were observed in various organs of pigeons infected with PiCV. This research, for the first time, identified the presence of inclusion bodies in the thyroid gland, esophagus, gizzard, and the third eyelid of pigeons infected with circovirus. Among the 164 dead pigeons examined, 96.95% (159/164) tested positive for PiCV.

Furthermore, the PiCV sequences found in this study exhibited a high similarity to those detected in European countries, suggesting a possible epidemiological association, potentially due to the importation of pigeons (31). In the histopathological results of the study of Raue et al. (2005), inflammation, hemosiderosis and inclusion bodies was observed in 19 cases (37%), 19 cases (37%), and 4 cases (7.8%), respectively, out of the 51 tested pigeons (22). The slightly higher prevalence of inclusion bodies in the study by Raue et al. compared to this study may be attributed to the phase of the disease in birds. As observed in their study, the presence of inclusion bodies was only seen in symptomatic pigeons. Interestingly, while 3 out of 6 asymptomatic pigeons tested positive for circovirus in the liver by PCR, none of these 6 pigeons exhibited inclusion bodies.

In 2002, Todd et al. conducted a comparative study in Belgium and Northern Ireland to evaluate four different methods for detecting circovirus in pigeons. The methods included PCR, in situ hybridization, dot blot hybridization, and histology. They found that PCR yielded the best results, as circovirus presence was confirmed in 84% of cases using PCR on bursa of Fabricius samples, followed by 75% with in situ hybridization, 63% with dot blot hybridization, and 41% with histology (32).

One possible reason for the higher reported percentage by Todd et al. could be related to the sampling from the bursa of Fabricius. Since circovirus is known to be an immunosuppressive agent, it leads to lymphoid depletion in the bursa of Fabricius, as previously explained. This interpretation is supported by the comparison of Todd's PCR results, where circovirus was detected in 84% of bursa of Fabricius samples and 56% of liver samples. Another reason could be attributed to the disease phase, which is highly effective in causing lesions. As explained previously, circovirus can remain hidden in tissues and emerge during immune system weakening. From this perspective, it can be understood why a 3.3% prevalence of lesions was observed in our histopathology study, while 100% circovirus detection was achieved through PCR.

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Authors' Contribution

Study concept and design: J.R, O.B.
Acquisition of data: O.B, R.K.
Analysis and interpretation of data: O.B., J.R., G.K., H.N, R.K.
Drafting of the manuscript: O.B.
Critical revision of the manuscript: J.R, H.N.
Statistical analysis: J.R, O.B.

Ethics

Ethical issues have been checked by all the authors.

Conflict of Interest

The authors declare no conflicts of interest.

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Data Availability

The data that support the findings of this study are available upon request from the corresponding author.

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