Histopathological and Molecular Detection of some Respiratory Diseases in backyard chickens around Ahvaz City

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Abstract

Introduction: Avian influenza virus subtype H9N2, Newcastle disease virus, and infectious bronchitis virus are among the major respiratory pathogens affecting chickens. Backyard chickens can serve as reservoirs for these diseases in industrial poultry. The present study aims to evaluate the molecular and histopathological investigation of H9N2, ND, and IB viruses in backyard chickens around Ahvaz city.

Material and Methods: 54 backyard chickens were purchased; after clinical examination, the birds were euthanized and necropsied. Following necropsy, specimens of the brain, trachea, lungs, and kidneys were obtained for molecular detection and histopathological evaluation. These birds showed emaciation and ruffled feathers.

Results: Investigation results showed that, 51% of the birds were positive for H9N2, 50% for ND virus, and 64% for IB virus. 85% of the birds were infected with one of the three viruses in the molecular analysis. Also, simultaneous infections with H9N2 and ND viruses were observed in

9%, H9 and IB viruses in 9%, ND and IB viruses in 4%, and all three viruses in 27% of birds. In the microscopic examination of tissue sections, meningoencephalitis and prevascular cuffing (PVC), interstitial lymphocytic nephritis, necrosis of the epithelial cells of urinary tubes, glomerulonephritis, lymphocytic tracheitis, interstitial lymphocytic pneumonia, bronchopneumonia, hyperplasia of lymphoid tissue around the bronchi, and enteritis were observed.

Conclusion: The results indicate widespread presence of respiratory viruses in backyard chicken. Considering that these birds had not received any vaccines according to their history, the molecularly positive samples indicate exposure to the viruses. It is concluded that backyard chickens serve as an essential reservoir of viruses and can contribute to their transmission to industrial poultry.

Keywords: Ahvaz city, backyard chicken, poultry viral reservoirs, respiratory viral infection

1. Introduction

Raising backyard chickens is common in Iran. Local poultry are among the most common domesticated animals worldwide and, due to their close contact with their surroundings, are among the most important sources of infectious diseases, posing a threat to the poultry farming industry. Respiratory problems in poultry farms may occur due to various viral, bacterial, and environmental factors [1]. Infection of backyard chickens with Newcastle disease virus (NDV), avian influenza virus (AIV), and infectious bronchitis virus (IBV) represents one of the most significant health and economic challenges to the poultry sector, particularly in rural and traditional farming systems [2, 3]. Backyard chickens often play an essential role in household food security and income generation; however, limited biosecurity measures and the absence of regular vaccination programs make these birds highly susceptible to infectious viral diseases [4]. The virus that causes Newcastle disease can infect more than 200 bird species. The virulent strains of Newcastle disease virus (NDV) are responsible for considerable economic losses in poultry industry worldwide. In developed countries, the outbreak of vND not only causes substantial economic losses but also imposes high costs on the poultry industry through disease control measures, such as vaccination. In many developing countries, vND is also endemic, thereby representing a major constraint on the growth and sustainability of the poultry industry.Newcastle disease is a highly contagious viral disease of poultry characterized by severe respiratory, nervous, and gastrointestinal symptoms. In unvaccinated flocks, mortality rates may reach up to 100% [2]. Backyard chickens act as important reservoirs for NDV due to their free-range management and frequent contact with other domestic and wild birds, thereby facilitating virus maintenance and transmission to commercial poultry farms [2, 5].

Avian influenza viruses (AIV) belong to the family Orthomyxoviridae and are classified into two major pathotypes based on their virulence in poultry, namely highly pathogenic avian influenza viruses and non-highly pathogenic avian influenza viruses.Avian influenza is a major viral disease affecting poultry worldwide. Low-pathogenic avian influenza (LPAI) serotypes such as H9N2 are widely prevalent in backyard poultry and can cause respiratory distress, decreased egg production, and immunosuppression [3, 4]. Highly pathogenic avian influenza (HPAI) strains can lead to severe outbreaks with high mortality rates. Moreover, certain avian influenza viruses pose a

potential zoonotic risk, raising concerns for public health [3]. The spread of H9N2 has been associated with factors such as migratory bird movements and the operation of live bird markets in some regions. Since the late 1990s, sporadic human infections with H9N2 have been reported in several Asian countries, prompting concerns regarding its potential public health implications [6]. There is also the possibility of virus transmission to humans, a change in virulence, and participation in genetic recombination with other influenza viruses[7].

Infectious bronchitis (IBV) is characterized as an acute viral infection with high transmissibility, involving the respiratory and urogenital systems of chickens of all ages. The disease has a global distribution, causes high morbidity, low mortality, and significant production losses. IBV infection can result in reduced growth performance, poor feed conversion, kidney damage, and reproductive disorders, especially in young or immunologically naïve birds [5]. The infectious bronchitis virus belongs to the family Coronaviridae, of which more than a dozen serotypes have been identified to date.The genetic diversity and frequent emergence of new IBV variants complicate disease control, particularly in backyard poultry populations with limited vaccination coverage [5].

Backyard chickens are often kept under minimal biosecurity conditions, which can lead to close contact with other birds, contaminated environments, and live bird markets. Consequently, these birds can serve as reservoirs and amplifiers of NDV, AIV, and IBV, increasing the risk of virus spread to industrial poultry operations [4, 8]. Co-infections with multiple respiratory viruses have been shown to exacerbate disease severity, leading to higher morbidity, mortality, and economic losses [8, 9]. Implementation of effective vaccination programs, improvement of biosecurity practices, and education of rural poultry keepers are essential strategies to reduce the circulation of these viruses in backyard chicken. Surveillance and early detection are also crucial to prevent spread to commercial farms and reduce public health risks [2, 4].

Therefore, considering the importance of the subject, the present study intends to examine the molecular and pathological contamination of backyard chickens in the Ahvaz region with Newcastle, influenza (H9N2), and infectious bronchitis viruses

2. Materials and Methods

2.1. Sampling

Fifty-four backyard chickens were randomly selected from different areas around Ahvaz city. The age, sex of the birds, clinical signs, and necropsy findings were recorded. Tissue samples of the trachea, lung, kidney, cecum, cecal tonsils, and brain were prepared for molecular and histopathological detection.

2.2. Molecular detection.

2.2.1. RNA extraction

After defrosting the tissue samples, adding 1000 µl of RNX (Cinnagen, Iran) 20 the Microtubes containing the 50 – 100 mg of pooled tissue samples from each bird, RNA was extracted according to the company's instructions Manufactured in the laboratory: it was kept at room temperature for 5 minutes until the cells were lysed and the nucleic acid was released. Thereafter, 200 µl of chloroform were added to each sample and shaken gently for 15 seconds at 5 °C for 5 minutes. The samples were then centrifuged for 15 minutes at 4 °C at 12,000 rpm. The supernatant containing the ribonucleic acid molecules was removed. In the next step, isopropanol was added with the same volume of this liquid to insolubilize the genome, and they were thoroughly mixed

and kept on ice for 15 minutes. The tubes were centrifuged again at 15 °C at 12,000 rpm for 15 minutes. After discarding the supernatant, 1 ml of 75% ethanol was added to each tube. The tubes were then centrifuged at 4 °C at 7500 rpm for 8 minutes. In the end, the supernatant was discarded and 50 µl of sterile DEPC water was added to each tube. The extracted RNAs were stored at -70 °C until cDNA production.

2.2.2. cDNA synthesis

To synthesize cDNA from the extracted RNA, a cDNA Synthesis Kit (manufactured by Yekta Tajhiz Azma Company, Iran) and a random hexamer general primer were used.

2.2.3. cDNA amplification

The reaction mixture was includsive of 2X Master Mix (with a concentration of 1.5 mM MgCl2, Amplicon, Canada) 10 µl, specific primers for the S gene to detect infectious bronchitis virus [10], the H gene to detect influenza virus serotype H9 [11], and the F gene to detect Newcastle disease virus [12], each 10 picomoles per µl (Table 1), template DNA 3 µl, water 6 µl in a final volume of 20 µl With temperature program. It is important to note that in every step of the PCR reaction, a negative control sample composed of DEPC water was used in place of DNA. Additionally, a positive control sample of RNA was extracted from the Massachusetts H120 and 4/91 strains of the infectious bronchitis vaccine. The positive controls for the Newcastle B1 and La Sota vaccines, as well as allantoic fluid containing the H9N2 virus for influenza, were also utilized (Table 2). The PCR products were analyzed by electrophoresis in a 1% agarose gel for 45 minutes at 100 V, then imaged after staining with a UV-safe dye.

Table 1. Nucleotide sequences of primers used in the PCR reaction

Agent	Target Gene site	Nucleotide Sequence
IBV	S1(728-749bp)	XCE1: CTC TAT AAA CAC CCT TAC A
	S1(1168-1193bp)	XCE2: CAC TGG TAA TTT TTC AGA TGG
AIV	H9(151-171bp)	F: CAC CTY ACA GAA CAC GG AAT
	H9(618-638bp)	R: GTC ACA CTT GTT GTT GTR TC
NDV	F(160-176bp)	F: GCAGCTGGCAGGGATTGTGGT
	F(483-503 bp)	R: TCTTTTGAGCGAGGATGTTG

Table 2. RT-PCR reaction cycles for NDV, H9N2 influenza and IBV

IBV		H9N2		NDV		Cycles
Duration	Temperature	Duration	Temperature	Duration	Temperature	
5 minutes	94 °c	5 minutes	94 °c	3 minutes	95 °c	Early Denaturation
30 seconds	94 °c	30 seconds	94 °c	30 seconds	95 °c	Denaturation
30 seconds	50 °c	30 seconds	50 °c	1 minutes	55 °c	Annealing
35 seconds	72 °c	35 seconds	72 °c	1 minutes	72 °c	Extension
4 minutes	72 °c	4 minutes	72 °c	10 minutes	72 °c	Final Extension
35 Cycle		35 Cycle		40 Cycle		Number of Cycles

2.3. Histopathology evaluation

Tissue samples were fixed in 10% buffered formalin. Tissue sections were prepared, and after going through the tissue preparation steps, including dehydration, clarification, and staining, they were cast. Several consecutive sections, 5 μm thick, from each block were stained with hematoxylin and eosin. After staining, the slides were mounted and examined under a light microscope.

3. Results

In this study, among 54 backyard chickens, 37 were hens and 17 were roosters, aged 3 months to 2 years. Clinical examination of these birds revealed nonspecific and general signs, including weight loss and ruffled feathers. After euthanasia, necropsy findings showed that most carcasses had hyperemic tracheae containing mucous or sometimes purulent exudate. The lungs and kidneys mainly appeared normal, though in some cases they were slightly congested. No necrotic lesions in the liver or thickening of the intestines were observed.

3.1. Molecular Results

46 (85%) cases of the examined birds were infected with at least one of the three viruses and in 8 birds we couldn't detect any viruses (Table 3, Figures 1-3). Positive cases occurred in both sexes and all age (3 months to 2 years) .

Table 3. RT-PCR Results

Agent	Positive Numbers (Percentage)	Co-Infections	Positive Numbers (Percentage)
Newcastle Disease Virus	27 (50%)	ND (only)	3 (11%)
		ND+AI	5 (18.5%)
		ND+IB	4 (14.8%)
Avian Influenza Virus	28 (52%)	AI (only)	3 (10.7%)
		AI+IB	5 (17.8%)
Infectious Bronchitis Virus	35 (65%)	IB (only)	11 (31.4%)
Negative birds	8(14.8%)		

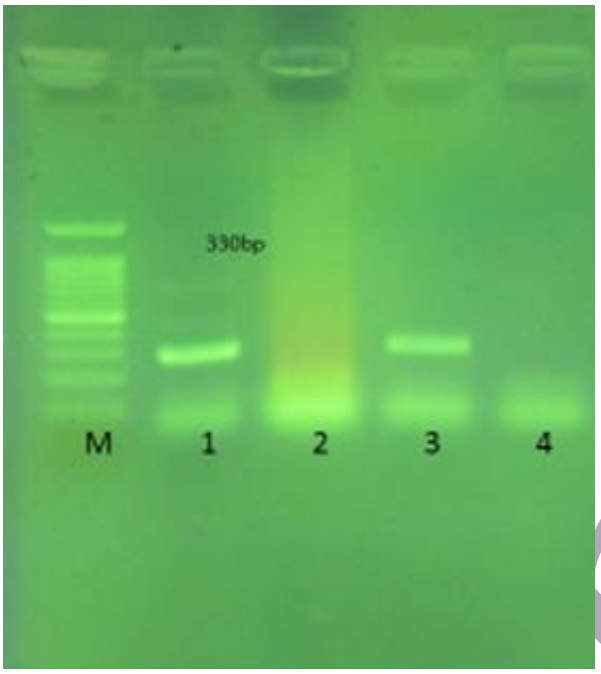

Figure 1. Electrophoresis of RT-PCR products using primers specific for the F gene of Newcastle disease virus (band size: 330 bp). M: DNA marker (100 bp); Lanes 1 and 2: tested samples; Lane 3: positive control; Lane 4: negative control.

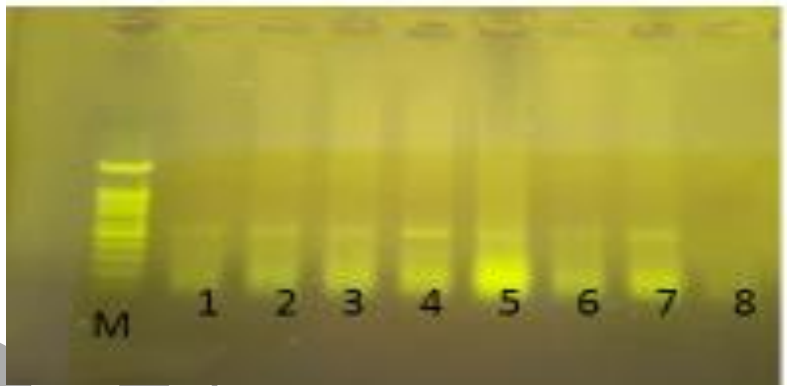

Figure 2. Electrophoresis of RT-PCR products using primers specific for the H9 gene of avian influenza virus (band size: 488 bp).M: DNA marker (100 bp); Lanes 1–6: tested samples; Lane 7: positive control; Lane 8: negative control

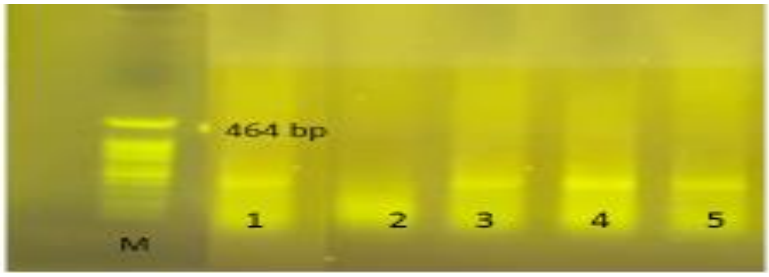

Figure 3. Electrophoresis of RT-PCR products using primers specific for the infectious bronchitis virus (IBV). M: DNA marker (100 bp); Lane 1: positive control; Lane 2: negative control; Lanes 3–5: tested samples.

3.2. Histopathologiacal Results

Microscopic examination of tissue sections from the brain, trachea, lungs, and kidneys intestines of various birds revealed different lesions, which are described below: **Brain:** Meningoencephalitis was diagnosed in 20 cases (Figure 4). **Kidney:** In birds infected with the infectious bronchitis virus or co-infections with all three viruses, interstitial lymphocytic nephritis, necrosis of tubular epithelial cells, and glomerulonephritis were observed (Figure 5). **Trachea:** In positive samples, tracheitis was observed. The lesions ranged from mild to severe (Figure 6). **Lungs:** In affected birds, interstitial lymphocytic pneumonia, bronchopneumonia, and hyperplasia of lymphoid tissue around the bronchi were observed (Figure 7).

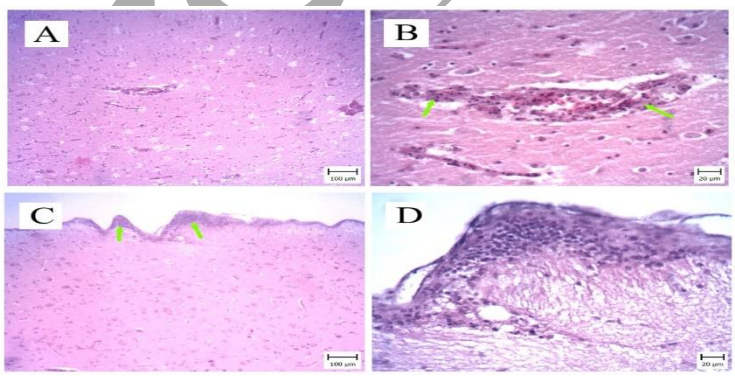

Figure 4. Brain section. A and B: Note the perivascular accumulation of astrogliar-type inflammatory cells (arrow). C and D: Note the presence of inflammatory cells beneath the pia mater (arrow) (H&E).

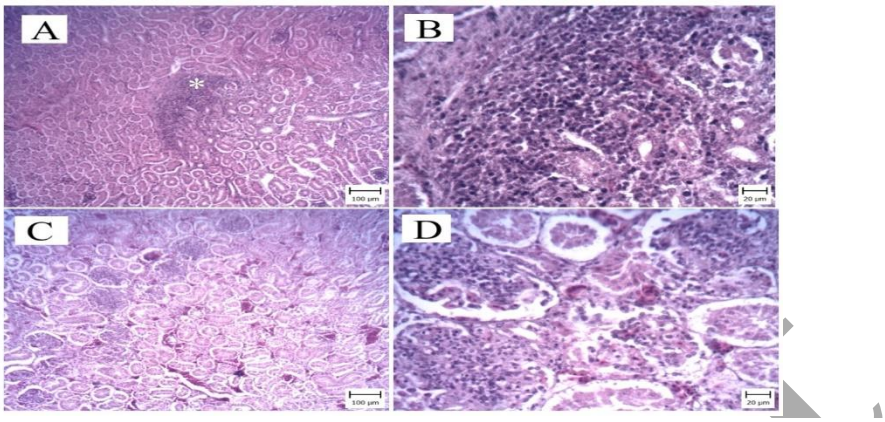

Figure 5. Kidney section. A and B: Interstitial nephritis. Note the accumulation of inflammatory cells among the tubules and glomeruli. C and D: Glomerulonephritis, with numerous cells and enlargement of the glomerular tuft (Hematoxylin and Eosin staining).

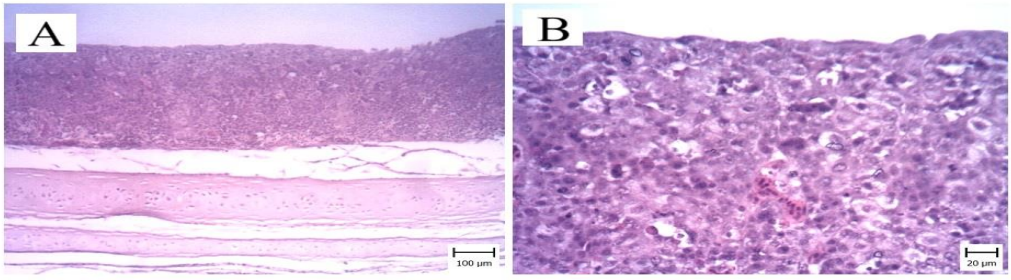

Figure 6. Trachea section. Severe tracheitis. Note the marked thickening of the tracheal mucosa due to the presence of inflammatory cells (Hematoxylin and Eosin staining).

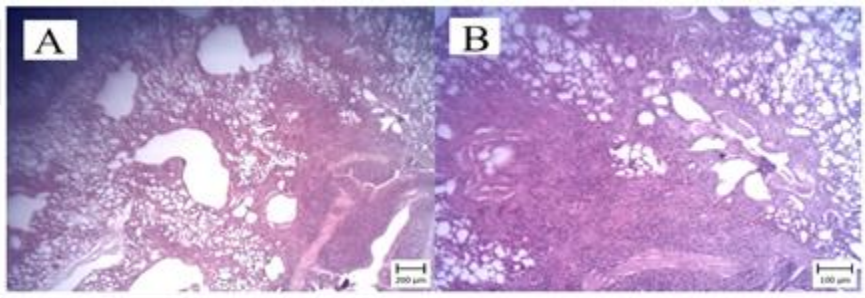

Figure 7. Lung section. Interstitial pneumonia. Note the accumulation of numerous inflammatory cells in the lung interstitial tissue (Hematoxylin and Eosin staining).

4. Discussion

Backyard chickens are usually kept in rural household yards as a source of protein and as part of the family's income. These birds are typically fed with table scraps and kitchen waste, kept in coops at night, and allowed to roam freely during the day. Because of their recognized role in the transmission and maintenance of various viral and bacterial pathogens, backyard birds have been frequently reported as infected hosts in many developing regions. In the present study, analysis of samples obtained from 54 backyard chickens revealed a high circulation of avian influenza H9, Newcastle disease, and infectious bronchitis viruses within this population. Reports similar to these have been documented by researchers worldwide. Broomand et al., in detecting influenza, Newcastle, and infectious bronchitis viruses in samples collected from 100 backyard chickens, demonstrated the widespread presence of these viruses among these birds. They found that even in cases where these birds had received inactivated influenza and Newcastle disease vaccines, the two viruses remained detectable. This indicates that this vaccine is unable to control the disease or prevent infection with wild strains in the field [13]. Sharma et al. screened backyard chicken flocks to determine the occurrence of Newcastle disease, avian influenza, and chicken infectious anemia viruses of West India and reported that serum samples were positive for antibodies against these agents in the blood, with different percentages [14]. The presence of Newcastle, influenza, infectious bronchitis ,and EDS viruses in rural poultry of Bangladesh was reported by Biswas et al. [15]. Shadmanesh and Mokhtari reported the presence of antibodies against Newcastle disease and avian influenza viruses, Mycoplasma gallisepticum, and Mycoplasma synoviale bacteria in rural Iranian poultry [16]. Mahzounieh et al. identified serological evidence of infectious bronchitis virus exposure in blood samples collected from rural chickens in Isfahan and reported that 85.3% of the sera tested were positive [17]. In 2021, Pouarbbas et al. conducted a serological study, molecular identification, and phylogenetic analysis of H9N2 avian influenza virus in domestic poultry in rural areas of Fars Province, Iran. The findings of this study showed that 54% of the serum samples were positive. The results of this study showed that, given the high positive serum titers in a large number of domestic poultry in rural areas, continuous monitoring of these birds for avian influenza infection and the implementation of more effective control programs seem necessary [18]. The high prevalence of IBV, H9, and NDV infections in the present study indicates the important role of these factors in causing respiratory disease in domestic poultry and in the spread of these viruses in broiler flocks around Ahvaz. In 2018, Amer et al. isolated and identified co-infections of H9N2 avian influenza and Newcastle disease in poultry flocks with

respiratory symptoms and mortality in Egypt. Natural co-infections with AIV H9N2 and velogenic ND (vNDV) viruses were identified [19]. In the microscopic examination of the lesions in the present study, lymphocytic interstitial nephritis, necrosis of epithelial cells of the urinary tract, glomerulonephritis, meningoencephalitis, and PVC, mild to severe lymphocytic tracheitis, lymphocytic interstitial pneumonia, and bronchopneumonia were observed. As noted in various sources, the microscopic lesions of these three viruses are not pathognomonic or specific, and they cannot be attributed to these infections with 100% certainty. However, because these tissue lesions were not observed in negative cases, they can be attributed to these viruses. The tracheal mucosa of chickens with infectious bronchitis virus becomes inflamed. Due to the loss of cilia, rounding and detachment of epithelial cells, and infiltration of heterophil cells and lymphocytes into the tissue, these changes are observed. Kidney damage caused by the infectious bronchitis virus primarily involves interstitial nephritis (inflammation of the tissue between the renal tubules). The virus causes degeneration, vacuolation, and desquamation of the tubular epithelium, and infiltration of heterophils into the interstitial tissue in the acute stages of the disease. Necrotic areas may also be seen, and in chronic cases, signs of attempted tubular epithelial repair are seen. During recovery, the inflammatory cell population changes to lymphocytes and plasma cells, and in some cases, destructive changes may lead to atrophy of various areas. In this study, 35 cases of infectious bronchitis that tested positive for molecular testing showed various microscopic findings: 11 cases showed interstitial nephritis, and 10 showed glomerulonephritis. Also, acute necrosis of the urinary tract was observed in 23 cases, as reported previously, which may be one of the causes of these injuries [20]. Gastrointestinal lesions are less common in mild infections of the infectious bronchitis virus. In a study by Raj and Jones, no pathological lesions were observed in the crop-to-ileum of chickens infected with the enterotropic G strain. However, there are reports of villous atrophy, congestion, and local infiltration of lymphocytes, macrophages, and heterophils in the rectum. In this study, all cases showed a spectrum of enteritis, which is certainly not solely due to viral agents but is more likely due to parasitic agents [21]. Newcastle disease microscopic apples are not pathognomonic and depend on many factors. The lymphoid tissues of the intestine, including cecal tonsils and Peyer's patches, are the primary sites of necrosis and hemorrhage in affected chickens and turkeys [22]. Virulent NDV infection in chickens and turkeys is commonly associated with lymphoid depletion and necrosis affecting the cecal tonsils, spleen, thymus, and bursa. Infection in chickens and turkeys may lead to hemorrhagic, ulcerative, or lymphocytic tracheitis. In this study, 14 cases of Newcastle disease patients showed lymphocytic tracheitis. Nine cases were also infected with two other viruses simultaneously. Newcastle disease virus also causes gliosis with perivascular cuffing (PVC) of inflammatory cells around the vessels of the cerebellum and brainstem, which is more common in acute forms. Another study investigated the prevalence of Newcastle disease in wild pigeons in São Paulo, Brazil, and found that the most significant histopathological changes were mononuclear cell infiltration in the brain, kidneys, ventricles, and spleen [23]. Low-pathogenic avian influenza viruses, such as serotype H9N2, cause the most common lesions in the respiratory tract in broilers; the tracheal mucosa may exhibit edema and congestion, with occasional evidence of hemorrhagic changes. Fibrinous-purulent bronchopneumonia can develop when secondary pathogens such as Pasteurella multocida or Escherichia coli are involved. In several natural cases in laying hens and in chickens infected intravenously, the kidneys were swollen and contained visceral urate deposits [24]. In this study,

18 cases of tracheitis were diagnosed: 14 were H9-positive and 9 were positive for all three agents. Low pathogenic avian influenza viruses cause a spectrum of pneumonia in poultry, ranging from fibrinous pneumonia to lymphocytic interstitial pneumonia. In acute and severe cases, fibrinous pneumonia with tracheitis is common. In this study, 19 cases of lymphocytic interstitial pneumonia were diagnosed. The predilection for the kidneys is dependent on the type of influenza virus. In fatal cases of LPAI infection, marked depletion of lymphocytes accompanied by necrosis or apoptotic changes has been observed in primary lymphoid organs, including the bursa of Fabricius, thymus, and spleen. In contrast, lymphocytic infiltration is frequently noted in other tissues such as the trachea and nasal mucosa. Detection of viral antigen in lymphocytes is uncommon, whereas strong expression is typically observed in necrotic epithelial tissues of the respiratory system, kidneys, and pancreas. Lymphocytic meningoencephalitis, characterized by focal gliosis, neuronal necrosis, and neurophagy, is commonly observed in severe influenza. In this study, 8 cases of meningoencephalitis were identified, all of which tested positive for all three viruses. However, the occurrence of some microscopic symptoms, such as meningoencephalitis and interstitial nephritis or interstitial pneumonia, may be caused by different viral agents.

Finally, it is concluded that the rural poultry in the Ahvaz region is an important reservoir of avian pathogenic viruses and can contribute to their transmission to industrial birds.

Conclusion

The present study demonstrates a high molecular prevalence of avian influenza H9N2, Newcastle disease virus, and infectious bronchitis virus in backyard chickens around Ahvaz city, with a considerable rate of mixed infections. The detection of single and co-infections, together with characteristic histopathological lesions in different tissues, indicates active circulation of these viruses in backyard poultry populations. These findings highlight the epidemiological significance of backyard chickens as important reservoirs for avian respiratory viruses and their potential role in transmitting infections to industrial poultry farms. Therefore, continuous surveillance, implementation of appropriate biosecurity measures, and inclusion of backyard flocks in control and prevention programs are essential to reduce the risk of virus spread and economic losses in the poultry industry.

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Authors' Contribution

Study concept and design: Z.B.
Conducting the experiment: A.G.
Analysis and interpretation of data: Z.B and A.R.
Drafting of the manuscript: S.F.
Critical revision of the manuscript: Z.B. and S.F.

Ethics

Ethical approval for this research was granted by the institutional Ethics Committee under approval number IR.SCU.REC.14030113.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Data Availability

All data generated are included in the current article.

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