

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

40 chickens serve as an essential reservoir of viruses and can contribute to their transmission to
41 industrial poultry.

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

43 **1. Introduction**

44 Raising backyard chickens is common in Iran. Local poultry are among the most common
45 domesticated animals worldwide and, due to their close contact with their surroundings, are among
46 the most important sources of infectious diseases, posing a threat to the poultry farming industry.
47 Respiratory problems in poultry farms may occur due to various viral, bacterial, and environmental
48 factors [1]. Infection of backyard chickens with Newcastle disease virus (NDV), avian influenza
49 virus (AIV), and infectious bronchitis virus (IBV) represents one of the most significant health and
50 economic challenges to the poultry sector, particularly in rural and traditional farming systems [2,
51 3]. Backyard chickens often play an essential role in household food security and income
52 generation; however, limited biosecurity measures and the absence of regular vaccination
53 programs make these birds highly susceptible to infectious viral diseases [4]. The virus that causes
54 Newcastle disease can infect more than 200 bird species. The virulent strains of Newcastle disease
55 virus (NDV) are responsible for considerable economic losses in poultry industry worldwide. In
56 developed countries, the outbreak of vND not only causes substantial economic losses but also
57 imposes high costs on the poultry industry through disease control measures, such as vaccination.
58 In many developing countries, vND is also endemic, thereby representing a major constraint on
59 the growth and sustainability of the poultry industry. Newcastle disease is a highly contagious viral
60 disease of poultry characterized by severe respiratory, nervous, and gastrointestinal symptoms. In
61 unvaccinated flocks, mortality rates may reach up to 100% [2]. Backyard chickens act as important
62 reservoirs for NDV due to their free-range management and frequent contact with other domestic
63 and wild birds, thereby facilitating virus maintenance and transmission to commercial poultry
64 farms [2, 5].

65 Avian influenza viruses (AIV) belong to the family Orthomyxoviridae and are classified into two
66 major pathotypes based on their virulence in poultry, namely highly pathogenic avian influenza
67 viruses and non-highly pathogenic avian influenza viruses. Avian influenza is a major viral disease
68 affecting poultry worldwide. Low-pathogenic avian influenza (LPAI) serotypes such as H9N2 are
69 widely prevalent in backyard poultry and can cause respiratory distress, decreased egg production,
70 and immunosuppression [3, 4]. Highly pathogenic avian influenza (HPAI) strains can lead to
71 severe outbreaks with high mortality rates. Moreover, certain avian influenza viruses pose a
72 potential zoonotic risk, raising concerns for public health [3]. The spread of H9N2 has been
73 associated with factors such as migratory bird movements and the operation of live bird markets
74 in some regions. Since the late 1990s, sporadic human infections with H9N2 have been reported
75 in several Asian countries, prompting concerns regarding its potential public health implications
76 [6]. There is also the possibility of virus transmission to humans, a change in virulence, and
77 participation in genetic recombination with other influenza viruses [7].

78 Infectious bronchitis (IBV) is characterized as an acute viral infection with high transmissibility,
79 involving the respiratory and urogenital systems of chickens of all ages. The disease has a global
80 distribution, causes high morbidity, low mortality, and significant production losses. IBV infection
81 can result in reduced growth performance, poor feed conversion, kidney damage, and reproductive

82 disorders, especially in young or immunologically naïve birds [5]. The infectious bronchitis virus
83 belongs to the family Coronaviridae, of which more than a dozen serotypes have been identified
84 to date. The genetic diversity and frequent emergence of new IBV variants complicate disease
85 control, particularly in backyard poultry populations with limited vaccination coverage [5].
86 Backyard chickens are often kept under minimal biosecurity conditions, which can lead to close
87 contact with other birds, contaminated environments, and live bird markets. Consequently, these
88 birds can serve as reservoirs and amplifiers of NDV, AIV, and IBV, increasing the risk of virus
89 spread to industrial poultry operations [4, 8]. Co-infections with multiple respiratory viruses have
90 been shown to exacerbate disease severity, leading to higher morbidity, mortality, and economic
91 losses [8, 9]. Implementation of effective vaccination programs, improvement of biosecurity
92 practices, and education of rural poultry keepers are essential strategies to reduce the circulation
93 of these viruses in backyard chicken. Surveillance and early detection are also crucial to prevent
94 spread to commercial farms and reduce public health risks [2, 4].

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

98 **2. Materials and Methods**

99 **2.1. Sampling**

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

104

105 **2.2. Molecular detection.**

106 **2.2.1. RNA extraction**

107 After defrosting the tissue samples, adding 1000 µl of RNX (Cinnagen, Iran) 20 the Microtubes
108 containing the 50 – 100 mg of pooled tissue samples from each bird, RNA was extracted according
109 to the company's instructions Manufactured in the laboratory: it was kept at room temperature for
110 5 minutes until the cells were lysed and the nucleic acid was released. Thereafter, 200 µl of
111 chloroform were added to each sample and shaken gently for 15 seconds at 5 °C for 5 minutes.
112 The samples were then centrifuged for 15 minutes at 4 °C at 12,000 rpm. The supernatant
113 containing the ribonucleic acid molecules was removed. In the next step, isopropanol was added
114 with the same volume of this liquid to insolubilize the genome, and they were thoroughly mixed
115 and kept on ice for 15 minutes. The tubes were centrifuged again at 15 °C at 12,000 rpm for 15
116 minutes. After discarding the supernatant, 1 ml of 75% ethanol was added to each tube. The tubes
117 were then centrifuged at 4 °C at 7500 rpm for 8 minutes. In the end, the supernatant was discarded
118 and 50 µl of sterile DEPC water was added to each tube. The extracted RNAs were stored at -70
119 °C until cDNA production.

120 **2.2.2. cDNA synthesis**

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

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125 **2.2.3. cDNA amplification**

126 The reaction mixture was inclusive of 2X Master Mix (with a concentration of 1.5 mM MgCl₂,
 127 Amplicon, Canada) 10 µl, specific primers for the S gene to detect infectious bronchitis virus [10],
 128 the H gene to detect influenza virus serotype H9 [11], and the F gene to detect Newcastle disease
 129 virus [12], each 10 picomoles per µl (Table 1), template DNA 3 µl, water 6 µl in a final volume of
 130 20 µl With temperature program. It is important to note that in every step of the PCR reaction, a
 131 negative control sample composed of DEPC water was used in place of DNA. Additionally, a
 132 positive control sample of RNA was extracted from the Massachusetts H120 and 4/91 strains of
 133 the infectious bronchitis vaccine. The positive controls for the Newcastle B1 and La Sota vaccines,
 134 as well as allantoic fluid containing the H9N2 virus for influenza, were also utilized (Table 2). The
 135 PCR products were analyzed by electrophoresis in a 1% agarose gel for 45 minutes at 100 V, then
 136 imaged after staining with a UV-safe dye.

137

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 GAR 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

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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

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141 **2.3. Histopathology evaluation**

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

147
148 **3. Results**

149 In this study, among 54 backyard chickens, 37 were hens and 17 were roosters, aged 3 months to
150 2 years. Clinical examination of these birds revealed nonspecific and general signs, including
151 weight loss and ruffled feathers. After euthanasia, necropsy findings showed that most carcasses
152 had hyperemic tracheae containing mucous or sometimes purulent exudate. The lungs and kidneys
153 mainly appeared normal, though in some cases they were slightly congested. No necrotic lesions
154 in the liver or thickening of the intestines were observed.

155 **3.1. Molecular Results**

156 46 (85%) cases of the examined birds were infected with at least one of the three viruses and in 8
157 birds we couldn't detect any viruses (Table 3, Figures 1-3). Positive cases occurred in both sexes
158 and all age (3 months to 2 years) .

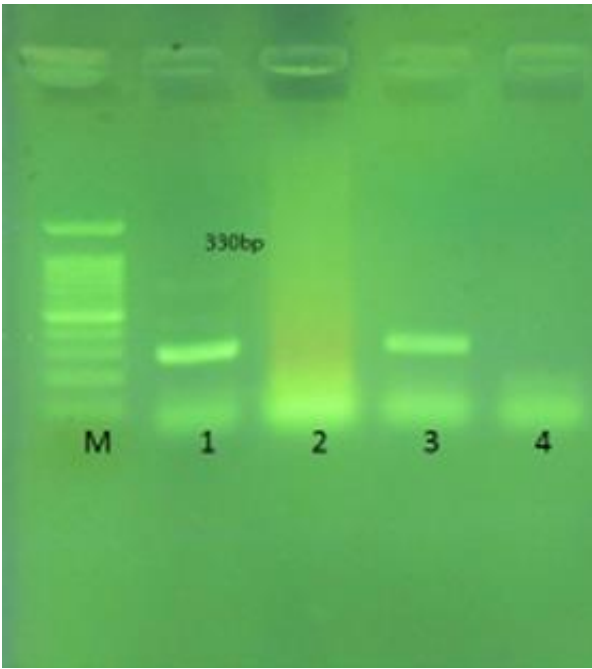
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Table 3. RT-PCR Results

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%)		

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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.

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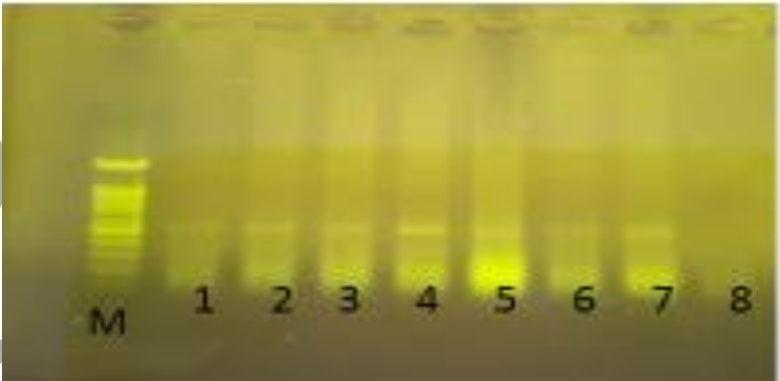


Figure 2. Electrophoresis of RT-PCR products using primers specific for the H9 gene of avian influenza virus (band size: 488 bp).M: DNA marker (100 bp); Lanes 1–6: tested samples; Lane 7: positive control; Lane 8: negative control

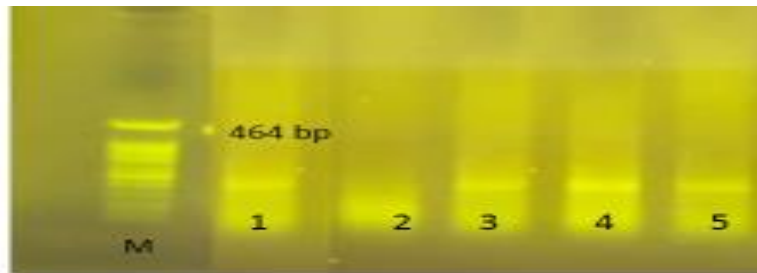


Figure 3. Electrophoresis of RT-PCR products using primers specific for the infectious bronchitis virus (IBV). M: DNA marker (100 bp); Lane 1: positive control; Lane 2: negative control; Lanes 3–5: tested samples.

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192 3.2. Histopathological Results

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

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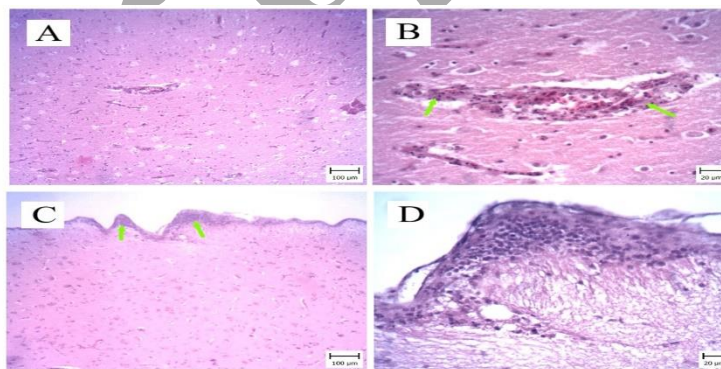
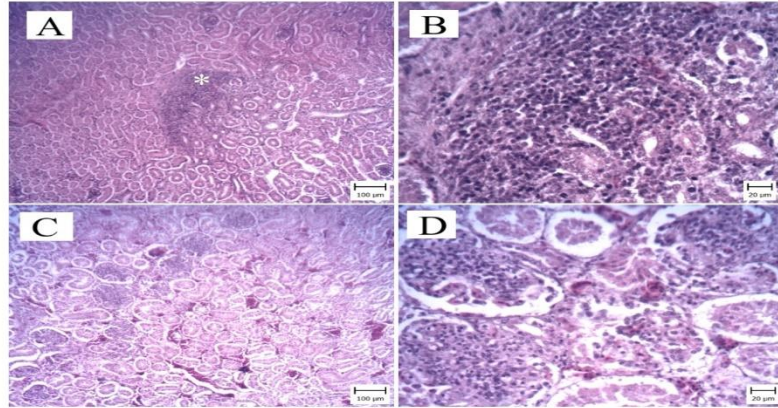


Figure 4. Brain section. A and B: Note the perivascular accumulation of astroglial-type inflammatory cells (arrow). C and D: Note the presence of inflammatory cells beneath the pia mater (arrow) (H&E).

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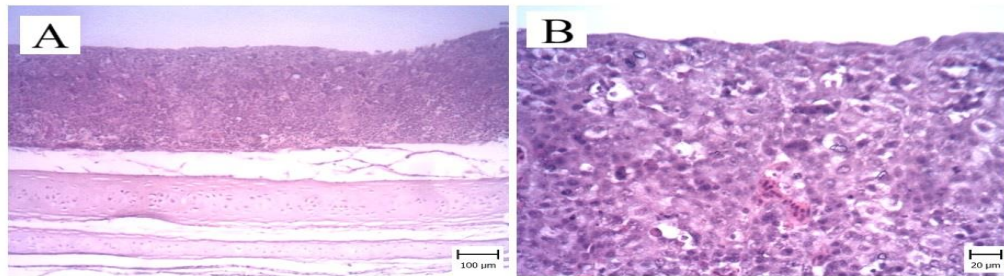
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211 **Figure 5.** Kidney section. A and B: Interstitial nephritis. Note the accumulation of inflammatory
212 cells among the tubules and glomeruli. C and D: Glomerulonephritis, with numerous cells and
213 enlargement of the glomerular tuft (Hematoxylin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

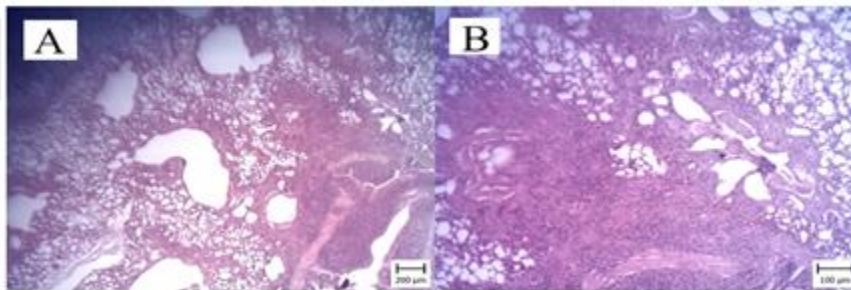
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220 **Figure 6.** Trachea section. Severe tracheitis. Note the marked thickening of the tracheal mucosa
221 due to the presence of inflammatory cells (Hematoxylin and Eosin staining).

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

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225 4. Discussion

226 Backyard chickens are usually kept in rural household yards as a source of protein and as part of
227 the family's income. These birds are typically fed with table scraps and kitchen waste, kept in
228 coops at night, and allowed to roam freely during the day. Because of their recognized role in the
229 transmission and maintenance of various viral and bacterial pathogens, backyard birds have been
230 frequently reported as infected hosts in many developing regions. In the present study, analysis of
231 samples obtained from 54 backyard chickens revealed a high circulation of avian influenza H9,
232 Newcastle disease, and infectious bronchitis viruses within this population. Reports similar to
233 these have been documented by researchers worldwide. Broomand et al., in detecting influenza,
234 Newcastle, and infectious bronchitis viruses in samples collected from 100 backyard chickens,
235 demonstrated the widespread presence of these viruses among these birds. They found that even
236 in cases where these birds had received inactivated influenza and Newcastle disease vaccines, the
237 two viruses remained detectable. This indicates that this vaccine is unable to control the disease or
238 prevent infection with wild strains in the field [13]. Sharma et al. screened backyard chicken flocks
239 to determine the occurrence of Newcastle disease, avian influenza, and chicken infectious anemia
240 viruses of West India and reported that serum samples were positive for antibodies against these
241 agents in the blood, with different percentages [14]. The presence of Newcastle, influenza,
242 infectious bronchitis, and EDS viruses in rural poultry of Bangladesh was reported by Biswas et
243 al. [15]. Shadmanesh and Mokhtari reported the presence of antibodies against Newcastle disease
244 and avian influenza viruses, *Mycoplasma gallisepticum*, and *Mycoplasma synoviale* bacteria in
245 rural Iranian poultry [16]. Mahzounieh et al. identified serological evidence of infectious
246 bronchitis virus exposure in blood samples collected from rural chickens in Isfahan and reported
247 that 85.3% of the sera tested were positive [17]. In 2021, Pouarbbas et al. conducted a serological
248 study, molecular identification, and phylogenetic analysis of H9N2 avian influenza virus in
249 domestic poultry in rural areas of Fars Province, Iran. The findings of this study showed that 54%
250 of the serum samples were positive. The results of this study showed that, given the high positive
251 serum titers in a large number of domestic poultry in rural areas, continuous monitoring of these
252 birds for avian influenza infection and the implementation of more effective control programs
253 seem necessary [18]. The high prevalence of IBV, H9, and NDV infections in the present study
254 indicates the important role of these factors in causing respiratory disease in domestic poultry and
255 in the spread of these viruses in broiler flocks around Ahvaz. In 2018, Amer et al. isolated and
256 identified co-infections of H9N2 avian influenza and Newcastle disease in poultry flocks with
257 respiratory symptoms and mortality in Egypt. Natural co-infections with AIV H9N2 and velogenic
258 ND (vNDV) viruses were identified [19]. In the microscopic examination of the lesions in the
259 present study, lymphocytic interstitial nephritis, necrosis of epithelial cells of the urinary tract,
260 glomerulonephritis, meningoencephalitis, and PVC, mild to severe lymphocytic tracheitis,
261 lymphocytic interstitial pneumonia, and bronchopneumonia were observed. As noted in various
262 sources, the microscopic lesions of these three viruses are not pathognomonic or specific, and they
263 cannot be attributed to these infections with 100% certainty. However, because these tissue lesions
264 were not observed in negative cases, they can be attributed to these viruses. The tracheal mucosa
265 of chickens with infectious bronchitis virus becomes inflamed. Due to the loss of cilia, rounding
266 and detachment of epithelial cells, and infiltration of heterophil cells and lymphocytes into the
267 tissue, these changes are observed. Kidney damage caused by the infectious bronchitis virus

268 primarily involves interstitial nephritis (inflammation of the tissue between the renal tubules). The
269 virus causes degeneration, vacuolation, and desquamation of the tubular epithelium, and
270 infiltration of heterophils into the interstitial tissue in the acute stages of the disease. Necrotic areas
271 may also be seen, and in chronic cases, signs of attempted tubular epithelial repair are seen. During
272 recovery, the inflammatory cell population changes to lymphocytes and plasma cells, and in some
273 cases, destructive changes may lead to atrophy of various areas. In this study, 35 cases of infectious
274 bronchitis that tested positive for molecular testing showed various microscopic findings: 11 cases
275 showed interstitial nephritis, and 10 showed glomerulonephritis. Also, acute necrosis of the urinary
276 tract was observed in 23 cases, as reported previously, which may be one of the causes of these
277 injuries [20]. Gastrointestinal lesions are less common in mild infections of the infectious
278 bronchitis virus. In a study by Raj and Jones, no pathological lesions were observed in the crop-
279 to-ileum of chickens infected with the enterotropic G strain. However, there are reports of villous
280 atrophy, congestion, and local infiltration of lymphocytes, macrophages, and heterophils in the
281 rectum. In this study, all cases showed a spectrum of enteritis, which is certainly not solely due to
282 viral agents but is more likely due to parasitic agents [21]. Newcastle disease microscopic apples
283 are not pathognomonic and depend on many factors. The lymphoid tissues of the intestine,
284 including cecal tonsils and Peyer's patches, are the primary sites of necrosis and hemorrhage in
285 affected chickens and turkeys [22]. Virulent NDV infection in chickens and turkeys is commonly
286 associated with lymphoid depletion and necrosis affecting the cecal tonsils, spleen, thymus, and
287 bursa. Infection in chickens and turkeys may lead to hemorrhagic, ulcerative, or lymphocytic
288 tracheitis. In this study, 14 cases of Newcastle disease patients showed lymphocytic tracheitis.
289 Nine cases were also infected with two other viruses simultaneously. Newcastle disease virus also
290 causes gliosis with perivascular cuffing (PVC) of inflammatory cells around the vessels of the
291 cerebellum and brainstem, which is more common in acute forms. Another study investigated the
292 prevalence of Newcastle disease in wild pigeons in São Paulo, Brazil, and found that the most
293 significant histopathological changes were mononuclear cell infiltration in the brain, kidneys,
294 ventricles, and spleen [23]. Low-pathogenic avian influenza viruses, such as serotype H9N2, cause
295 the most common lesions in the respiratory tract in broilers; the tracheal mucosa may exhibit
296 edema and congestion, with occasional evidence of hemorrhagic changes. Fibrinous-purulent
297 bronchopneumonia can develop when secondary pathogens such as *Pasteurella multocida* or
298 *Escherichia coli* are involved. In several natural cases in laying hens and in chickens infected
299 intravenously, the kidneys were swollen and contained visceral urate deposits [24]. In this study,
300 18 cases of tracheitis were diagnosed: 14 were H9-positive and 9 were positive for all three agents.
301 Low pathogenic avian influenza viruses cause a spectrum of pneumonia in poultry, ranging from
302 fibrinous pneumonia to lymphocytic interstitial pneumonia. In acute and severe cases, fibrinous
303 pneumonia with tracheitis is common. In this study, 19 cases of lymphocytic interstitial pneumonia
304 were diagnosed. The predilection for the kidneys is dependent on the type of influenza virus. In
305 fatal cases of LPAI infection, marked depletion of lymphocytes accompanied by necrosis or
306 apoptotic changes has been observed in primary lymphoid organs, including the bursa of Fabricius,
307 thymus, and spleen. In contrast, lymphocytic infiltration is frequently noted in other tissues such
308 as the trachea and nasal mucosa. Detection of viral antigen in lymphocytes is uncommon, whereas
309 strong expression is typically observed in necrotic epithelial tissues of the respiratory system,
310 kidneys, and pancreas. Lymphocytic meningoencephalitis, characterized by focal gliosis, neuronal

311 necrosis, and neurophagy, is commonly observed in severe influenza. In this study, 8 cases of
312 meningoencephalitis were identified, all of which tested positive for all three viruses. However,
313 the occurrence of some microscopic symptoms, such as meningoencephalitis and interstitial
314 nephritis or interstitial pneumonia, may be caused by different viral agents.

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

317

318 **Conclusion**

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

329

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333

334 **Authors' Contribution**

335 Study concept and design: Z.B.

336 Conducting the experiment: A.G.

337 Analysis and interpretation of data: Z.B and A.R.

338 Drafting of the manuscript: S.F.

339 Critical revision of the manuscript: Z.B. and S.F.

340

341 **Ethics**

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

344

345 **Conflict of Interest**

346 The authors declare that they have no conflict of interest.

347

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351

352 **Data Availability**

353 All data generated are included in the current article.

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