Concurrent infection of *Avibacterium paragallinarum* and fowl adenovirus in layer chickens

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ABSTRACT The diagnosis of a concurrent infection of *Avibacterium paragallinarum* and fowl adenovirus (**FAdV**) in an infectious coryza–like outbreak in the outskirt of Beijing is reported. The primary signs of the infection were acute respiratory signs, a drop in egg production, and the presence of hydropericardium–hepatitis syndrome–like gross lesions. Laboratory examination confirmed the presence of *A. paragallinarum* by bacterial isolation and a species-specific PCR test. In addition, conventional serotyping identified the isolates as Page serovar A. Fowl adenovirus was isolated from chicken liver specimen and identified by *hexon* gene amplification. In addition, histopathologic analysis and transmission electron microscopy examination further confirmed the presence of the virus. Both *hexon* gene sequencing and phylogenetic analysis defined the viral isolate as FAdV-4. The pathogenic role of *A. paragallinarum* and FAdV was evaluated by experimental infection of specific-pathogen-free chickens. The challenge trial showed that combined *A. paragallinarum* and FAdV infection resulted in more severe clinical signs than that by FAdV infection alone. The concurrent infection caused 50% mortality compared with 40% mortality by FAdV infection alone and zero mortality by *A. paragallinarum* infection alone. To our knowledge, this is the first report of *A. paragallinarum* coinfection with FAdV. The case implies that concurrent infections with these 2 agents do occur and more attention should be given to the potential of multiple agents during disease diagnosis and treatment.

Key words: *Avibacterium paragallinarum*, fowl adenovirus, concurrent infection, infectious coryza (IC), hydropericardium-hepatitis syndrome (HHS)

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INTRODUCTION

Infectious coryza (**IC**), caused by *Avibacterium paragallinarum* is a widely occurring respiratory disease of chickens that results in acute inflammation in the upper respiratory tract, nasal discharge, facial edema, and conjunctivitis. The disease causes growth retardation in broilers and egg drop in layers, particularly in multiage farms (Blackall and Soriano-Vargas, 2020). Isolates of *A. paragallinarum* can be serotyped by 2 inter-related schemes—the Page scheme that recognizes serovars A, B, and C (Page, 1962) and the Kume scheme that recognizes 3 serogroups (A, B, and C) and nine serovars (A-1 to A-4, B-1, and C-1 to C-4) (Kume et al., 1983; Blackall et al., 1990a). Both schemes are based on hemagglutination inhibition tests (Kume et al., 1983; Blackall et al., 1990b) and the Kume serogroups correspond to the Page serovars (Blackall et al., 1990a). *A. paragallinarum* has been circulating in China for more than 3 decades (Chen et al., 1993), and the dominant serovars currently are Page serovars A and B (Wang et al., 2016b; Sun et al., 2018).

Infectious coryza is usually characterized by low mortality and high morbidity, but the impact of the disease becomes much greater when other pathogens are involved, such as coinfections with *Gallibacterium anatis* (Paudel et al., 2017), *Ornithobacterium rhinotracheale* (Morales-Erasto et al., 2016), avian influenza virus (Kishida et al., 2004), or stress factors associated with poor environmental conditions (Han et al., 2016). Recently, an unusual clinical case with severe neurologic signs and relevant histologic changes of IC was reported

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in commercial broilers in California, and *A. paragallinarum* and infectious bronchitis virus were isolated (Crispo et al., 2018). A synergistic effect between *A. paragallinarum* and infectious bronchitis virus was suggested as resulting in exacerbation of clinical signs and increased mortality (Crispo et al., 2018).

Fowl adenovirus (**FAdV**), a nonenveloped dsDNA virus, is classified as group I avian adenovirus in the genus *Aviadenovirus* and the family Adenoviridae, and it has 12 serotypes (Hess, 2000). Fowl adenovirus is transmitted horizontally by mechanical means or the oral–fecal route (Abdul-Aziz and Hassan, 1995; Chandra et al., 2000) and vertically from parent to progeny through eggs (Hafez, 2011; Grafl et al., 2012). Although the pathogenic impact of most FAdV isolates is still questionable, the role of the virus as a primary respiratory pathogen in immunocompromised birds and in the presence of respiratory pathogens and other organisms has been recognized (Gowthaman et al., 2012; Eregae et al., 2014; Meng et al., 2018). Hepatitis–hydropericardium syndrome (**HHS**) caused by FAdV is a serious and emerging disease occurring in many countries of the world where chickens are raised under intensive conditions (Pan et al., 2017a; Shah et al., 2017; Schachner et al., 2018). In recent years increased outbreaks of the disease have been reported in many provinces across China (Zhao et al., 2015; Xia et al., 2017; Niu et al., 2018). Hepatitis–hydropericardium syndrome outbreaks caused by FAdV-4 (serotype 4) have resulted in severe epidemics with 30–100% mortalities in several areas of China since 2015 (Pan et al., 2017a). It has been shown that strains circulating in China before 2014 and after 2015 had different ancestors and that new strains found in China were derived from earlier Indian strains, with some genetic differences (Zhang et al., 2016).

Here, we report on an investigation into an outbreak of IC-like disease along with a FAdV concurrent infection, as well as providing the details of a follow-up challenge trial.

MATERIALS AND METHODS

Field Data

In December 2018, an IC-like outbreak occurred in a 30-wk-old Roman layer flock near Beijing. The clinical features described were swollen faces, respiratory symptoms, excretion of green-to-yellow feces, nearly 25% egg drop, and 40% mortality. Five sick birds were sent to our laboratory in Beijing for examination.

Bacterial Isolation and Identification

Caseous or mucosal materials were aseptically collected from both infraorbital sinuses of 5 sick birds with swabs and streaked onto tryptic soy agar plates supplemented with 10% chicken serum and 0.0025% NAD. The plates were incubated at 37°C in a candle jar for 18–24 h (Page, 1962; Rimler and Davis, 1977). Suspect *A. paragallinarum* colonies (tiny dewdrop-like colonies showing iridescence under obliquely transmitted light of 40 × dissecting microscope) were subcultured 3 times for purification and stored at −80°C in 30% glycerol.

The suspect *A. paragallinarum* isolates were identified by a simple biochemical test—catalase test (Blackall and Soriano-Vargas, 2020) and by the *A. paragallinarum* Haemophilus paragallinarum-2 PCR as previously described (Chen et al., 1996). Page serovar A, B, and C reference strains (221, 0,222, and Modesto, respectively) were used as positive controls.

These isolates were then examined in the Page scheme serotyping by the hemagglutination inhibition test (Eaves et al., 1989). Rabbit antisera against Page serovar A, B, and C reference strains (221, 0,222, and Modesto), prepared earlier in this laboratory using methods previously reported (Kume et al., 1983), were used. Hemagglutinins of the 3 reference strains (as positive controls) and the isolates were prepared by potassium thiocyanate and sonication treatment as previously described (Eaves et al., 1989).

In addition, the total DNA was prepared from collected sinus swabs using the TIANamp blood, cell and tissue genomic DNA kit (Tiangen, China) as per the manufacturer's instructions, followed by PCR examination for the presence of *Mycoplasma gallisepticum* and *O. rhinotracheale* using previously described protocols (Umali et al., 2018; Felice et al., 2020). Meanwhile, Haemophilus paragallinarum-2 PCR (Chen et al., 1996) was conducted on these DNA samples. The enlarged liver samples were subjected to bacterial culture using tryptic soy agar with 5% chicken serum and Luria-Bertani agar. Both agars were incubated under aerobic and microaerophilic conditions for 24 h at 37°C.

Virus Isolation

After the 5 sick chickens were sacrificed and gross lesion inspected, liver tissue from a HHS suspect chicken was collected aseptically and homogenized in 3 × volumes of PBS (0.01 M, pH7.2), containing penicillin (100 IU/mL) and streptomycin (100 μg/mL) followed by 3 freeze/thaw cycles, then centrifuged at 20,000 × *g* for 10 min. The supernatant was filtered through a 0.22-μm membrane, and 0.2 mL was inoculated via yolk sac into each of five 7-day-old chicken embryos. The eggs were candled daily for 10 d. The embryos that died within 24 h were discarded, while allantoic fluid from those died embryos between 24 h to 10 d were harvested, pooled, and blindly transferred for 5 generations. Allantoic fluid from the dead embryos of the last passage was harvested, pooled, and stored at −80°C for further use.

Histologic Analysis

A portion of swollen liver tissue from the HHS-suspect chicken was fixed in 10% neutral-buffered formalin for

histopathologic examination. The samples were progressively dehydrated, embedded in paraffin, cut into 5-mm sections, and stained with hematoxylin and eosin. The liver tissue sections were observed under a microscope (Leica, Germany).

Transmission Electron Microscopy

Liver tissue sections from the chicken with hepatomegaly were incubated with 2.5% glutaraldehyde (electron microscopic grade) for 40 min, and the supernatant was then replaced with fresh fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH7.4) and held overnight at room temperature. Then, the fixative was replaced with 8% sucrose in 0.1 M Sorensen's phosphate buffer (pH7.4; Electron Microscopy Sciences, Inc.) for 20 min. The cell pellets were processed for transmission electron microscopy observation. Images were obtained using a transmission electron microscope (**TEM**) (JEOL JEM-1230), and the negative contrast technique were used to identify FAdV.

Fowl Adenovirus PCR and Sequencing

DNA from the liver tissue and pooled allantoic fluid of embryos was extracted using a DNA extraction kit (Omega Bio-Tek, Norcross, GA) as per the manufacturer's instructions. A PCR was based on a FAdV-specific primer pair that targeted a 667-bp partial *hexon* gene (F: 5′-CAACTACATCGGGTTCAGGGATAACTTC-3′ and R: 5′-CCAGTTTCTGTGGTGGTTGAAGGGGTT-3′) (Ye et al., 2016). The PCR program was as follows: initial denaturation for 5 min at 95°C, 35 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 2 min and a final extension step of 10 min at 72°C. A known FAdV strain was used as a positive control and sterilized water and liver DNA from an specific-pathogen-free (**SPF**) chicken as negative controls. The PCR products were visualized in 1% agarose gels.

The PCR products were sent for sequencing, and the assembled sequence was analyzed by BLAST in GenBank. The serotype was determined by a phylogenetic analysis. The phylogenetic tree was generated by the neighbor-joining method using MEGA7 (Kumar et al., 2016). Bootstrap values were calculated from 1,000 replicates of the alignment.

Embryo Lethal Dose Determination of Isolated Virus

The fifth generation of isolated virus was 10-fold serially diluted with PBS, and 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions of the virus were inoculated into allantoic cavity of five 7-day-old SPF chicken embryos, (0.2 mL dose), which were then incubated at 37°C for 10 d. The embryos that died within 24 h were discarded. At day 10, pathologic changes in the dead embryos were observed and recorded. The Reed-Muench method was used to calculate the embryo lethal dose (**ELD_{50}**) of the virus (Reed and Muench, 1938).

Experimental Infection of A. paragallinarum and FAdV

An experimental infection was performed in fourty 3-wk-old SPF leghorn chickens. Three-week-old chickens were selected as birds of this age have been shown to be appropriate for experimental infections with FAdV-4 (Wang et al., 2016a). In addition, chickens of all ages are susceptible to *A. paragallinarum* infection (Blackall and Soriano-Vargas, 2020). The handling of birds was in accordance with the Guidelines of Animal Care and Use Committee of the Institute under the approval of Institute of Animal Husbandry and Veterinary Medicine (Permit number: 2014–05). All efforts were made to alleviate animal suffering and to improve their quality of life. The birds were randomly divided into 4 groups of 10 birds housed in separate isolation facilities, with feed and water provided ad libitum. Group 1 was inoculated with an *A. paragallinarum* isolate via the infraorbital sinus. The dosage given was 0.2 mL of 10^7 cfu/mL, a typical dose used for the experimental reproduction of IC (Reid and Blackall, 1984). Group 2 was inoculated by breast muscle injection with 0.1 mL, 10^3 ELD_{50} of the isolated FAdV, again a typical experimental challenge dose (Wang et al., 2016a). Group 3 was infected by both *A. paragallinarum* and FAdV isolates, with same doses and routes as for groups 1 and 2. Group 4 was injected saline as negative control. All birds were monitored daily, and an individual score was given based on clinical signs: 0—active with no adverse clinical sign; 1—mild facial swelling and/or nasal discharge, slightly weak with dropped wings; 2—moderate facial swelling and/or nasal discharge, weak, apathetic, ruffled feathers, and diarrhea; 3—severe facial swelling and/or nasal discharge, unable to move or stand, breathing intensely with eyes closed. Euthanasia was applied to birds clinically rated the highest score (3), and these birds were recorded as dead.

At day 7, all the remaining birds were euthanized by injecting sodium pentobarbital. Swabs taken from infraorbital sinuses from groups 1, 3, and 4 were streaked onto blood agar plates with a *Staphylococcus epidermidis* nurse colony (Soriano et al., 2004). The plates were incubated overnight at 37°C with 5% CO_2. The presence of satellitic colonies, indicating recovery of the challenge strain of *A. paragallinarum*, was recorded. Cloacal swabs taken from groups 2, 3, and 4 were used to detect FAdV by the PCR described previously (Ye et al., 2016).

Statistical Analysis

For the challenge trial, clinical signs scores were recorded and compared by Fisher's least significant difference test. The mortality was analyzed by chi-square test. The values in each row with different superscript letters are significantly different ($P < 0.05$).

RESULTS

Postmortem examination revealed mucus in the infraobital sinus of the 5 birds, very similar to that

seen in IC infections (Blackall and Soriano-Vargas, 2020). No gross lesions in the trachea, lungs, and air sacs were found. The pale and enlarged liver and pericardial effusion found in 1 bird were very similar to the lesions caused by HHS as described elsewhere (Shah et al., 2017; Wang and Zhao, 2019).

Isolation and Identification of Bacteria From Sinus Samples

A total of 5 chickens that showed IC signs were examined for the presence of *A. paragallinarum* and other pathogenic bacteria. All of 5 chickens yielded catalase-negative hemophilic organisms. The suspected colonies were positive in the *A. paragallinarum* species–specific PCR. *A. paragallinarum* was the dominant bacterium, and no other significant bacteria were recovered. In addition, the sinus swabs were all negative for *M. gallisepticum* and *O. rhinotracheale* but positive for *A. paragallinarum* by PCR. In conventional serotyping, the *A. paragallinarum* isolates were found to be Page serovar A.

Bacterial Culture of Liver Samples

No bacteria were isolated from the liver samples.

Fowl Adenovirus Isolation and Identification

In the process of FAdV isolation, infected chicken embryos showed stunted growth, hemorrhagic, and yellow-white necrosis on the liver and with turbid allantoic fluid. The titer of the virus in the finally harvested allantoic fluid was determined as $6 \times 10^{4.5}$ ELD_{50}/mL.

PCR detection with DNA extracted from both the original HHS-like chicken liver and the harvested allantoic fluid samples resulted in the 0.7-kb band expected for FAdV *hexon* gene. The analysis of the PCR products by sequencing and BLAST in GenBank confirmed that FAdV *hexon* gene–specific fragments were obtained. The isolate was named as 19-C1. The partial *hexon* gene sequence of 19-C1 was deposited in GenBank (accession number MK894431). The sequence was 100% identical with that of multiple serotype 4 FAdV isolates, and it was most closely related to SD1601/FAdV (accession number MH006602), a recognized Chinese serotype 4 strain (Figure 1).

The virus did not have the ability to agglutinate red blood cells from the chicken, duck, mouse, rabbit and sheep and was acid-resistant, alkali-resistant, and heat-sensitive.

Pathologic Outcomes and TEM Observation of FAdV

In this study, hydropericardium effusion and hepatomegaly were recognized at necropsy in the HHS-suspect chicken from the field and the birds in the experimental infection (Figure 2). Histopathologic analysis exhibited intranuclear inclusion body hepatitis and clear basophilic inclusions in the nuclei of hepatocytes within necrotic lesions (Figure 3).

Examination of the field chicken liver by TEM revealed the existence of multiple nonenveloped, icosahedral viral particles. These virions were aggregated and indicated crystal lattice formations in the nuclei of hepatocytes (Figure 4).

Experimental Infections with A. paragallinarum and FAdV

The typical clinical signs of both diseases were observed from day 2 after challenge. In *A. paragallinarum*–infected group 1, all the chickens showed facial swelling (one sided or bilaterally) and nasal discharge. However, there was no mortality. *A. paragallinarum* was recovered from every bird in this group. In FAdV-challenged group 2, almost every chicken showed depression, ruffled feathers, respiratory distress, and anorexia

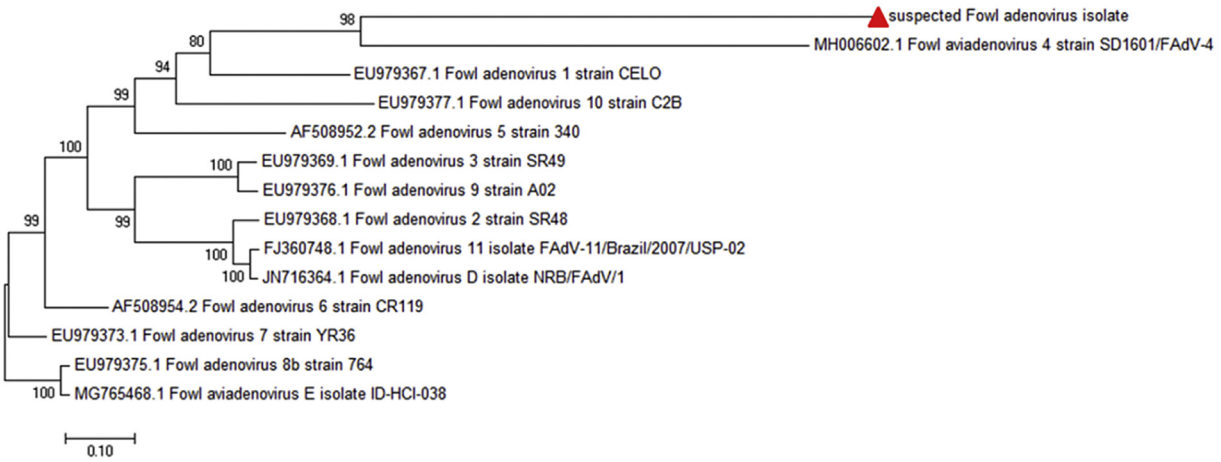

Figure 1. The phylogenetic relationship based on neighbor-joining analysis of hexon gene sequences from FAdV reference strains and the isolate from this study. The numbers at nodes indicate bootstrap value obtained from 1,000 resamplings. The scale bar represents sequence variation. The reference sequence of FAdV-4 (serotype 4) is at the same branch with the isolate. Abbreviation: FAdV, fowl adenovirus.

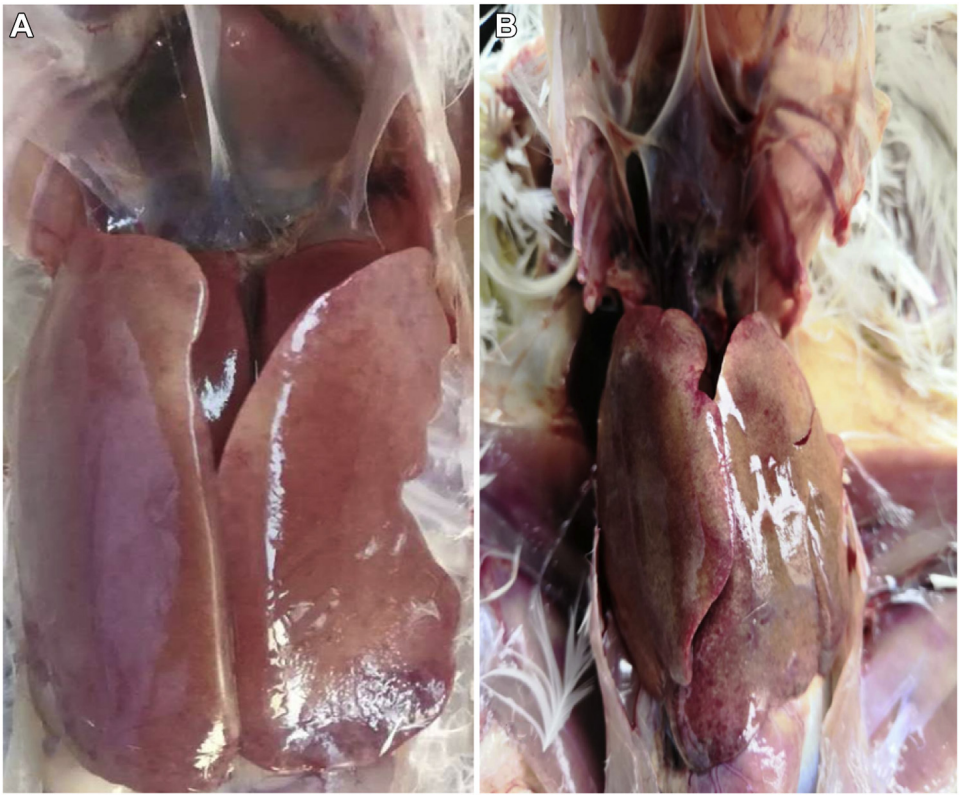

Figure 2. Autopsy changes of the chicken with FAdV infection. (A) Hydropericardium and hepatomegaly of field case; (B) Hepatomegaly and focal necrosis of experimental infection bird. Abbreviation: FAdV, fowl adenovirus.

from day 2 onward. At day 4, four birds clinically reached the highest clinical signs score (3) and were euthanized immediately. At the end of the trial, the infection rate for this group was 10 of 10, by both clinical signs as well as by the *hexon* gene PCR assay applied to cloacal swab samples. In FAdV and *A. paragallinarum* coinfected group 3, 10 of 10 birds showed clinical signs as observed in groups 1 and 2, and 5 of 10 birds were euthanized within 4 d after challenge. The *hexon* gene PCR was positive for all 10 birds. In addition, *A. paragallinarum* was reisolated from each bird. In contrast, there were no clinical signs found in the control group, the PCR assay for FAdV was negative and no *A. paragallinarum* was isolated (Table 1). Formal statistical analysis found no significant difference between the mean clinical signs score for the *A. paragallinarum* group and the FAdV and *Av. paragallinarum* coinfected group. Both of these groups had significantly more clinical signs than the FAdV group. The mortality seen in the FAdV and *A. paragallinarum* coinfected group and the FAdV group was not significantly different.

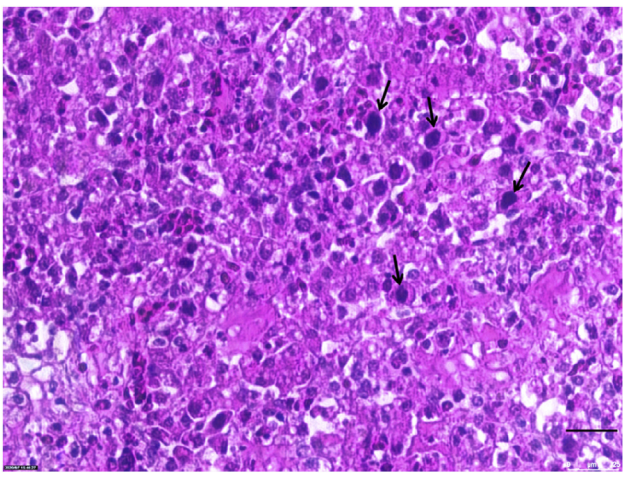

Figure 3. Histopathologic changes in FAdV-infected chicken liver from field case: Basophilic inclusion bodies, H&E. Scale bar: 25 μm. Abbreviations: FAdV, fowl adenovirus; H&E, hematoxylin and eosin.

DISCUSSION

Respiratory diseases are responsible for major economic losses in the poultry industry (Sid et al., 2015). Nowadays, it is common to see disease outbreaks linked to more than a single agent (Umar et al., 2018). In this study, five *A. paragallinarum* isolates and a FAdV-4 strain were isolated from diseased layer chickens in an IC-HHS co-infection outbreak. By PCR, we excluded *M. gallisepticum* and *O. rhinotracheale* from involvement in the respiratory infection. In addition, no other significant bacterial pathogen was recovered from the sinus samples.

Based on clinical appearance and gross lesions, the liver sample from the only HHS-suspect chicken was subjected to FAdV isolation, histopathology and morphology examination, and ELD_{50} titration of the virus. The serotype of the isolate 19-C1 was determined by

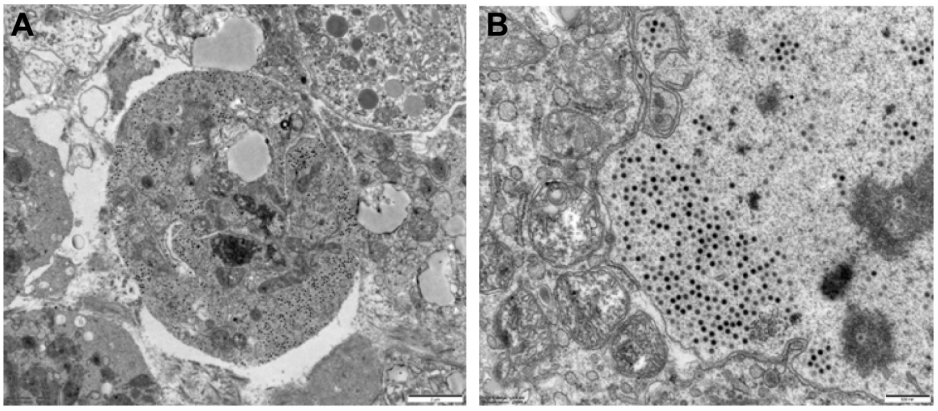

Figure 4. TEM of the field chicken liver. (A, B) Virions were collected in the nuclei of hepatocytes. Scale bar in (A) 2.0 μm, Scale bar in (B) 0.5 μm.

partial hexon gene amplification, sequencing, and phylogenetic analysis. The synergistic infection by the isolated bacterium and virus was evaluated through animal experiment in SPF chickens.

When performing the necropsy of the FAdV-challenged birds, lesions in the liver and heart were inspected and found (data not shown), but no recording of lung lesions was performed. This was an unfortunate situation as lung lesions have been linked with FAdV (Shah et al., 2017; Mo et al., 2019). In addition, Gowthaman et al. (2012) had detected FAdV in 13 of 34 commercial poultry farms with respiratory disease complex by RT-PCR/PCR from tissue samples and suggested that the virus should be considered as a primary respiratory pathogen. There is additional evidence that suggests that respiratory infections may occur with FAdV. Mo et al. (2019) compared 3 challenge routes for FAdV-4 infection in chickens (intramuscular, intranasal, and oral injection) and found that intranasal injection could cause a high mortality (up to 50%). In addition, they detected FAdV-4 DNA in tissues (liver, heart, lung) and oropharyngeal and cloacal swabs of the 3 route infected birds by RT-PCR, suggesting that airborne transmission may be a transmission route for FAdV-4 infection. Li et al. (2019) also by experiment demonstrated FAdV-4 can be transmitted among chickens by aerosols. Conventionally, *A. paragallinarum* and FAdV-4 would be regarded as targeting 2 different body systems—the respiratory tract *for Av. paragallinarum* and the heart and digestive organs for FAdV-4. Based on the literature cited previously, it would appear that FAdV-4 may be an agent which can be involved in diseases linked with multiple different organs or systems in chickens.

Cowen (1988) had shown that an effective and practical way to propagate type I avian adenoviruses of 11 serotypes in SPF chicken embryos was via yolk sac injection and observing gross and microscopic lesions or death at 9 d after injection. Others have used chicken embryos for FAdV isolation from clinical samples (Shah et al., 2017; Wang et al., 2019). In our work, although the *hexon* gene could be amplified by PCR from infected

Table 1. Results of clinical sign scores,[1] morbidity, mortality and reisolation rates[2] of the groups inoculated with *Avibacterium paragallinarum* (Avpg) and PCR detection of fowl adenovirus (FAdV).

	Group (inoculum)			
Day after challenge	1 (Avpg)	2 (FAdV)	3 (Avpg and FAdV)	4 (saline)
1	1.6[1]	0	1.8	0
2	2.0	0.3	2.0	0
3	2.0	1.7	2.1	0
4	2.0	2.0	2.2	0
5	1.6	1.3	2.0	0
6	1.1	1.0	1.8	0
7	0.8	0.8	1.6	0
Total mean score ± SD	1.59 ± 0.48[a]	1.01 ± 0.72[b]	1.93 ± 0.21[a]	0[c]
Morbidity[#]	10/10	10/10	10/10	0/10
Mortality	0[b]	4/10[a]	5/10[a]	0/10[b]
Avpg reisolation	10/10	ND	10/10	0/10
FAdV-PCR	ND	10/10	10/10	0/10

Avpg reisolation, number of chickens Avpg reisolated/the total number of chickens in the group; FAdV-PCR, number of chickens FAdv-PCR positive/the total number of chickens in the group; Morbidity, number of diseased chickens/the total number of chickens in the group; Mortality, number of chickens killed/the total number of chickens in the group.

Abbreviation: ND, not done.

[1]Data are given as mean values of clinical signs in each group.

[2]Values in each row with different superscript letters are significantly different ($P < 0.05$).

embryos, few embryos were found dead by day 5. The incubation time then was extended till day 10, when most embryos were dead. Others have reported delayed FAdV isolation. As an example, Mo et al. (2019) succeeded in FAdV isolation and propagation by 3 passages in embryonated eggs plus 10 consecutive passages in Chicken embryo kidney cell cultures.

The gross lesions, histopathology, and TEM images (Figures 2–4) reported in this study are resembling that of other relevant reports (Hess, 2000; Shah et al., 2017; Meng et al., 2019). Clearly, the liver is a key tissue for FAdV synthesis and package. The constitution and lattice formation of these viral particles further confirmed the diagnosis of FAdV infection.

Artificial infection with *A. paragallinarum* alone does not normally cause death of the birds (Reid and Blackall, 1984; Jacobs et al., 2003; Paudel et al., 2017) only shows typical clinical signs, as it occurred here in group 1, that is highest score was (2). Combined *A. paragallinarum* and FAdV infection caused more severe clinical signs than that by FAdV infection alone. The difference of clinical scores between group 2 and group 3 was statistically significant, which indicated that concurrent infection aggravated the symptoms of FAdV infection alone group. Group 2 also showed significant difference with group 1, whereas there were no significant difference between group 1 and group 3, this might be due to the clinical signs appeared were IC signs, while the FAdV infection was developed as a subclinical infection in these chickens, which did not gave much clinical features, and could only be found by immunological detection (Li et al., 2019). The level of mortality found in group 2 and group 3 showed no significant difference and was similar to that seen in the field outbreak (40% mortality). Fowl adenovirus-4 infection could cause high mortality, but the visible symptoms in chickens were relatively mild at incubation period and rehabilitation phase. Clinical recovery of the birds in groups 2 and 3 occurred at day 6 after infection and was manifested by a reduction in the severity of clinical signs (data not shown). The virus could still be detected in cloaca at the end of the trial (day 7), and it might persist for a long time. It has been reported -that after infection of 7- to 35-day-old SPF chickens with FAdV-4, the virus could be detected from chicken oropharyngeal and cloacal swabs at 35 and 40 d later, at levels of upto 33.3% (Mo et al., 2019). Moreover, this trial confirms that the quantity of virus shed in the feces of birds does not correlate with the pathogenicity of an individual strain (Matos et al., 2016).

At present, several inactivated FAdV-4 vaccines have been reported in China (Pan et al., 2017b; Du et al., 2017; Meng et al., 2019). However, there is no combined vaccine for *A. paragallinarum* and FAdV-4 yet. The prevention and control of these 2 diseases in combination has to be achieved by using effective monovalent vaccines for both diseases, improving environmental hygiene and ensuring good biosecurity and nutrition for layer flocks.

In conclusion, *A. paragallinarum* is an important bacterial respiratory pathogen of chickens and FAdV-4 is a novel emerging viral agent, both causing significant economic losses to the poultry industry throughout China and around world. There has no earlier report to describe concurrent infection of *A. paragallinarum* and FAdV-4. Further studies on the mechanism and impact of the concurrent infection involving IC and HHS are required.

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DISCLOSURES

The authors declare no conflict of interest.

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