Characterization of emergent *Avibacterium paragallinarum* strains and the protection conferred by infectious coryza vaccines against them in China

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ABSTRACT Infectious coryza (**IC**), an acute respiratory disease of chickens, is caused by *Avibacterium paragallinarum*. Here, the current epidemiological status of IC was investigated in China over 5 yr (2013 to 2018). A total of 28 *Av. paragallinarum* field isolates were identified by PCR tests and by sequence analysis of the hemagglutinin gene. The pathogenicities of 4 field isolates, the efficacy of 2 commercial inactivated oil-emulsion IC vaccines and vaccines containing different *Av. paragallinarum* isolates were also evaluated. The PCRs revealed a high rate (51.5%) of sample positivity for *Av. paragallinarum* during 2013 to 2018. Phylogenetic analysis showed that most field strains fell into the same cluster and had a farther genetic relationship with the early isolates from China. Pathogenicity testing revealed that the Chinese *Av. paragallinarum* isolates were able to induce the typical clinical signs of IC; hence, they were clearly pathogenic to chickens. Vaccine efficacy tests revealed that the 2 commercial inactivated oil-emulsion IC vaccines we tested had low protection rates against 2 selected *Av. paragallinarum* isolates after a single immunization, whereas the inactivated vaccine containing the *Av. paragallinarum* BJ26 isolate generated a relatively high protection rate against the field isolates compared with other three tested vaccines. The results indicate that IC is currently prevalent in China, and that commercial vaccines have not counteracted its presence in this country.

Key words: infectious coryza, *Avibacterium paragallinarum*, hemagglutinin antigen gene, pathogenicity, vaccine

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INTRODUCTION

Avibacterium paragallinarum is a pathogen responsible for an acute respiratory disease in chickens. This disease, infectious coryza (**IC**), which is characterized by sneezing, nasal discharge, facial swelling and conjunctivitis, causes significant economic losses to the global chicken farming industry via growth retardation and reduced egg production (Blackall, 1999). *Avibacterium paragallinarum* isolates can be serotyped by 2 interrelated schemes: the Page scheme, which recognizes serovars A, B, and C, and the Kume scheme, which recognizes three serogroups (A, B, and C) and 9 hemagglutinin serovars (A-1, A-2, A-3, A-4, B-1 and C-1, C-2, C-3, C-4) (Page, 1962; Kume et al., 1983; Blackall et al., 1990a; Sakamoto et al., 2013). Both schemes use hemagglutination-inhibition testing to type *Av. paragallinarum*.

The hemagglutinin antigen (**HA**) plays an important role in the pathogenicity and immunogenicity of *Av. paragallinarum*. The nucleotide sequences of the HA genes are conserved among the 11 serotyped reference strains, which include 0083(A-1), 0222(B-1) and Modesto(C-2) (Hobb et al., 2002). This suggests that HA is a common antigen among *Av. paragallinarum* serovars. Hemagglutinin antigen proteins from the Page-type serovars A and C have been shown to be protective antigens (Sawata et al., 1982; Takagi et al., 1991). Chickens immunized with the purified HA antigen are protected from challenge infections with *Av. paragallinarum* (Noro et al., 2008; Wu et al., 2011). Therefore, HA is important for pathogen virulence and host protective immunity against *Av. paragallinarum* and is, therefore, considered to be a potential component of subunit vaccines against IC.

The presence of *Av. paragallinarum* has been reported in many countries such as China, USA, Indonesia, India and UK in recent years (Zhang et al., 2003; Welchman et al., 2010; Patil et al., 2017; Crispo et al., 2018; Wahyuni et al., 2018). Many researchers in different parts of the world work on the pathogenicity of *Av. paragallinarum* (Sawata and Kume, 1983;

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Blackall, 1999). Major differences have been reported among the 9 serovars of the 3 *Av. paragallinarum* serogroups, and H-18 (serovar C-1) has been shown to be the most virulent strain of the nine studied reference strains of this species (Soriano et al., 2004b). Similar severe lesions have been observed in birds inoculated with either H-18 or ESV-135 strains (Trujillo-Ruíz et al., 2016). Investigations in Uganda have confirmed that serogroup C strains of *Av. paragallinarum* isolates are pathogenic to chickens (Byarugaba et al., 2007). In general, no cross-protection has been seen between Page serovars and Kume serogroups, good cross-protection has been seen among serogroup A isolates, partial cross-protection has been seen among serovar B isolates, and lower cross-protection has been seen between serogroup C isolates (Yamaguchi et al., 1991; Blackall, 1999; Soriano et al., 2004a).

In China, all 3 serovars are recognized: serovar A was first reported in 1987, serovar C was first reported in 1995, and serovar B was first reported in 2003 (Feng, 1987; Lin et al., 1995; Bragg, 2002; Zhang et al., 2003). Recently, outbreaks of IC have been increasingly reported in different Chinese provinces, including in vaccinated flocks. The aims of the current study were to investigate the prevalence of *Av. paragallinarum* during disease outbreaks in poultry using a PCR test, analyze the characteristics of the HA genes, compare the pathogenicity of field isolates, and evaluate the protection conferred by commercial vaccines and two experimental monovalent vaccines made from Chinese isolates.

MATERIALS AND METHODS

Current Epidemiological Status of IC in China

A total of 56 samples were collected from 14 Chinese provinces between 2013 and 2018. The samples were from chickens that showed the clinical sign of facial swelling. The pathogen identity in the samples was confirmed as *Av. paragallinarum* by PCR testing with a pair of primers (forward: 5′-GCGTCAGTAGCA CAAGCT-3′; reverse: 5′-TTTAACTGAGATTTCTAC ACG-3′) based on the nucleotide sequence of the *Av. paragallinarum* H-18 strain's polymerase gene available in GenBank (Accession No. AF491823). The PCRs specifically amplified a 500-bp fragment of the polymerase gene. The primers were synthetized by Sangon Biotech (Shanghai, China).

Bacterial Isolation and Identification

The samples collected in this study were swabs from the infraorbital sinuses of the chickens. They were used to inoculate tryptic soy broth agar (**TSA**) plates (supplemented with 5% chicken serum and 0.0025% nicotinamide adenine dinucleotide). The plates were incubated for 24 h at 37°C with 5% CO_2. Suspected colonies of *Av. paragallinarum* were then cultured on 5% blood agar plates, and *Staphylococcus aureus* was added to the TSA as a colony feeder. After incubation, satellite growth on the plates was examined.

Sequence Analysis of the HA Gene

The *Av. paragallinarum* HA gene was amplified using PCR assay. The primer pair (forward: 5′-TGAGG GTAGTCTTGCACGCGAAT-3′; reverse: 5′-CAAGGT ATCG ATCGTCTCTCTACT-3′) was designed based on the Modesto strain's sequence (GenBank Accession No. AF491827) to amplify the HA gene sequences from *Av. paragallinarum* isolates. PCR products with the expected 969-bp fragment length were directly sequenced. All the sequences obtained from the 28 Chinese field samples were submitted to GenBank (Table 1). A phylogenetic tree of the HA gene sequences was constructed using the 16 sequences already published in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using the DNASTAR software suite (version 7.1, DNASTAR, Madison, WI, USA) and MEGA4.1 tools (Molecular Evolutionary Genetics Analysis, version 4.1).

Animals and Ethics Statement

Specific-pathogen-free (**SPF**) White Leghorn chickens (5 or 9 wk old) were purchased from Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd. (Beijing, China). All animals used in this study were cared for in accordance with the experimental protocols, and all procedures, including the possibility of animal death without anaesthetics, were specifically considered and approved by the Animal Welfare and Ethical Censor Committee of China Agricultural University.

Pathogenicity Tests

A total of 50 SPF chickens, 9 wk of age, were randomly allocated into 5 groups of 10 chickens each, with each group placed in a single isolator. Four bacterial isolates HeB19, HuN22, BJ26, and BJ28, were used. The first 4 groups were challenged by infraorbital sinus inoculation with 0.2 ml of the inoculum (1×10^8 colony-forming units (**CFU**) per ml), from the 4 field isolates that had been cultured for 8 h in TSB. The fifth group, the control, was inoculated the same way with 0.2 ml of sterilized TSB (Table 2). Clinical signs of IC were recorded from the second to the eleventh day post-inoculation. The presence and degree of nasal discharge and facial swelling in the infection-challenged chickens were scored according to the following scale as previously reported; specifically, 0: no clinical signs; 1: mild signs (slight facial swelling and nasal discharge); 2: moderate signs (moderate facial swelling and nasal discharge); and 3: severe signs (severe facial swelling, abundant nasal discharge, and lacrimation) (Bragg, 2002).

Table 1. Detailed description of *Av. paragallinarum* isolates involved in clinical outbreaks of infectious coryza.

Number	Strain	Date	Origin	Type of chicken	Age (wk)	Coryza vaccine[1]	Signs[2]	Accession number
1	BJ02	03/2014	Beijing	Broiler breeder	43	Yes	+	MN080795
2	LN03	10/2014	Liaoning	Broiler breeder	45	Yes	+	MN080796
3	SX04	01/2015	Shanxi	Layer breeder	22	Yes	+	MN080791
4	BJ05	04/2015	Beijing	Layer	23	No	+	MN080769
5	BJ06	05/2015	Beijing	Layer	22	Yes	+	MN080778
6	SX07	06/2015	Shanxi	Broiler breeder	72	Yes	+	MN080792
7	TJ08	07/2015	Tianjin	Layer	18	Yes	+	MN080770
8	BJ09	10/2015	Beijing	Layer	37	No	+	MN080776
9	JX10	02/2016	Jiangxi	Broiler breeder	28	Yes	+	MN080773
10	BJ11	04/2016	Beijing	Layer breeder	57	Yes	+	MN080777
11	BJ12	06/2016	Beijing	Layer	40	Yes	+	MN080779
12	GX14	01/2017	Guangxi	Broiler breeder	28	Yes	+	MN080794
13	TJ15	02/2017	Tianjin	Layer	35	Yes	+	MN080790
14	HeB16	02/2017	Hebei	Layer breeder	50	Yes	+	MN080789
15	HeB17	02/2017	Hebei	Broiler breeder	53	Yes	+	MN080793
16	HeB18	03/2017	Hebei	Broiler breeder	35	Yes	+	MN080783
17	HeB19	03/2017	Hebei	Layer breeder	33	Yes	+	MN080781
18	HeB20	03/2017	Hebei	Layer breeder	38	Yes	+	MN080771
19	BJ21	03/2017	Beijing	Layer	20	No	+	MN080780
20	HuN22	04/2017	Hunan	Layer	31	Yes	+	MN080782
21	HeB23	04/2017	Beijing	Broiler breeder	26	Yes	+	MN080772
22	BJ25	07/2017	Beijing	Layer	36	Yes	+	MN080784
23	BJ26	12/2017	Beijing	Broiler breeder	34	Yes	+	MN080786
24	BJ27	03/2018	Beijing	Broiler breeder	31	Yes	+	MN080775
25	BJ28	04/2018	Beijing	Broiler breeder	33	Yes	+	MN080774
26	BJ29	06/2018	Beijing	Layer	36	Yes	+	MN080785
27	BJ30	06/2018	Beijing	Layer	34	Yes	+	MN080787
28	BJ31	07/2018	Beijing	Layer	35	Yes	+	MN080788

[1]The vaccination program varied between farms.
[2]Clinical signs were judged by veterinary experts.

The total disease score for each group was calculated by dividing the mean daily disease score by the days of observation. Infraorbital sinus swabs from all chickens at 10 D post challenge (dpc) were streaked on blood agar and crossed with an *S. aureus* feeder strain. After incubation for 24 h at 37°C with 5% CO_2, the satellite growth in the plates was examined.

Vaccine Preparation

Two experimental vaccines were made from the liquid media cultures of HuN22 and BJ26 strains. The experimental vaccines contained at least 1×10^9 CFU/ml of each *Av. paragallinarum* isolate. Two commercial IC vaccines containing a bivalent inactivated oil-emulsion vaccine of types A and C and a trivalent inactivated oil-emulsion vaccine of types A, B, and C, were purchased directly from the market.

Immunization and Challenge Infection Tests

Chickens (110 SPF, 5 wk of age) were divided into 6 groups. The first 4 groups of 20 chickens were vaccinated with the 2 commercial vaccines and 2 experimental vaccines, separately. The vaccines were given as a single 0.5 ml dose by the subcutaneous or intramuscular route. The operation was conducted in accordance with the manufacturer's instructions. The fifth and sixth group were not vaccinated and served as positive and negative controls, respectively. On day 28 post-vaccination, 10 chickens from each of the vaccinated groups and from the positive control group were challenged by infraorbital sinus inoculation with 0.2 ml of the HuN22 or BJ26 strains containing 1.5×10^8 CFU/mL of each strain separately. The negative control group was inoculated in the same way but with 0.2 ml of sterilized TSB instead (Table 3). The same scoring system and bacterial re-isolation method were used in this challenge test. If the immunized chickens showed any clinical signs of facial swelling or nasal discharge during the observation period that defined the status of the vaccine as non-protective.

Statistical Analysis

Statistics were analyzed in GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, California, USA). One-way ANOVA was used for comparison of morbidity, and incidence score in different groups. Significance was reported for all analyses for $P < 0.05$.

RESULTS

Prevalence Survey

During 2013 to 2018, 56 samples from suspected outbreaks of IC were evaluated by PCR testing for birds that showed signs of acute upper respiratory tract infections like coughing, nasal discharge and facial edema. From the samples, 31/56 (55.4%) were positive for *Av.*

Table 2. Experimental design and results of the pathogenicity evaluation of *Av. paragallinarum* isolates in chickens.[1]

Group	Number of chickens	Age (wk)	Challenge strain	Infection route	Infection dose	Average score value	No. of diseased chickens[2]	Morbidity	Bacterial re-isolation[3]	Bacterial re-isolation rate
A	10	9	HeB19	Infra-orbital sinus injection	2×10^7CFU/0.2 mL	0.95^{A}	9/10	90^{A}	3/10	30
B	10	9	HuN22	Infra-orbital sinus injection	2×10^7CFU/0.2 mL	1.55^{B}	10/10	100^{A}	3/10	30
C	10	9	BJ26	Infra-orbital sinus injection	2×10^7CFU/0.2 mL	0.82^{C}	10/10	100^{A}	2/10	20
D	10	9	BJ28	Infra-orbital sinus injection	2×10^7CFU/0.2 mL	0.37^{D}	6/10	60^{A}	1/10	10
E	10	9	Medium	Infra-orbital sinus injection	0.2 mL	0^{E}	0/10	0^{B}	0/10	0

[1]Means with different superscripts are significantly different ($P < 0.05$).
[2]Number of diseased chickens/no. of chickens in group.
[3]Number of positive samples from cultures/no. of chickens detected.

Table 3. Experimental design and results of immunization and challenge tests against *Av. paragallinarum* isolates in chickens.[1]

Group	No. of chickens	Age (wk)	Vaccine	Route and dose of inoculation	Challenge	Route and dose of challenge	Average score value	Bacterial re-isolation (%)	Morbidity (%)	Protection (%)[5]
A	10	5	Vaccine 1[2]	Subcutaneous injection, 0.5 mL	HuN22	Infra-orbital sinus injection, 3×10^7 CFU/0.2 mL	1.00^{A}	0/10 (0)	10/10 (100)^{A}	0/10 (0)
B	10	5	Vaccine 1	Subcutaneous injection, 0.5 mL	BJ26	Infra-orbital sinus injection, 3×10^7 CFU/0.2 mL	0.90^{a}	0/10 (0)	10/10 (100)^{a}	0/10 (0)
C	10	5	Vaccine 2[3]	Intramuscular injection, 0.5 mL	HuN22	Infra-orbital sinus injection, 3×10^7 CFU/0.2 mL	0.74^{B}	0/10 (0)	7/10 (70)^{B}	3/10 (30)
D	10	5	Vaccine 2	Intramuscular injection, 0.5 mL	BJ26	Infra-orbital sinus injection, 3×10^7 CFU/0.2 mL	0.56^{b}	1/10 (10)	8/10 (80)^{ab}	2/10 (20)
E	10	5	HuN22[4]	Intramuscular injection, 0.5 mL	HuN22	Infra-orbital sinus injection, 3×10^7 CFU/0.2 mL	0.86^{C}	4/10 (40)	6/10 (60)^{B}	4/10 (40)
F	10	5	HuN22	Intramuscular injection, 0.5 mL	BJ26	Infra-orbital sinus injection, 3×10^7 CFU/0.2 mL	0.89^{a}	1/10 (10)	10/10 (100)^{a}	0/10 (0)
G	10	5	BJ26[4]	Intramuscular injection, 0.5 mL	HuN22	Infra-orbital sinus injection, 3×10^7 CFU/0.2 mL	0.77^{B}	3/10 (30)	6/10 (60)^{B}	4/10 (40)
H	10	5	BJ26	Intramuscular injection, 0.5 mL	BJ26	Infra-orbital sinus injection, 3×10^7 CFU/0.2 mL	0.50^{b}	1/9 (11)	5/9 (56)^{b}	4/9 (44)
I	10	5	/	/	HuN22	Infra-orbital sinus injection, 3×10^7 CFU/0.2 mL	1.58^{D}	3/10 (30)	10/10 (100)^{A}	0/10 (0)
J	10	5	/	/	BJ26	Infra-orbital sinus injection, 3×10^7 CFU/0.2 mL	0.85^{a}	0/10 (0)	10/10 (100)^{a}	0/10 (0)
K	10	5	/	/	Medium	Infra-orbital sinus injection, 0.2 mL	0.00	0/10 (0)	0/10 (0)	10/10 (100)

[1]Means with different superscripts are significantly different ($P < 0.05$).
[2]A commercial bivalent inactivated vaccine for coryza (A + C).
[3]A commercial trivalent inactivated vaccine for coryza (A + B + C).
[4]The experimental vaccine was prepared as water-in-oil emulsions using HuN22 or BJ26 *Av. paragallinarum* isolates.
[5]A protected chicken was defined as a chicken that exhibited no clinical signs during the observation period. Protection indicates the number of chickens exhibiting no clinical signs out of the total number of birds.

Table 4. The results of screening for infectious coryza in disease-suspected field samples by PCR.[1]

Date	Number of samples tested (n)	Number of samples found positive for *Av. Paragallinarum*[2] (n)	Prevalence (%)
08/2013-12/2013	3	1	33.3
01/2014-12/2014	5	2	40.0
01/2015-12/2015	8	6	75.0
01/2016-12/2016	7	4	57.1
01/2017-12/2017	21	13	61.9
01/2018-09/2018	12	5	41.7

[1]PCR = Polymerase chain reaction.
[2]*Av. paragallinarum* = *Avibacterium paragallinarum*.

paragallinarum. Observed by each year, the positivity rate was found to show an upwards trend over the 5-yr period (Table 4). The results indicate IC is currently prevalent in China.

Isolation and Identification

From the 31 PCR-positive samples, 28 *Av. paragallinarum* strains were isolated using TSA and blood agar media, the detailed information for which is shown in Table 1. The *Av. paragallinarum* isolates produced smooth colonies, as characterized by tiny dewdrops in the media with no hemolysis. All isolates needed the supply of additional nicotinamide adenine dinucleotide.

Sequence Analysis of the HA Gene

According to the phylogenetic analysis we conducted based on the HA encoding gene, the phylogenetic tree indicates that the isolated strains belong to a new branch (Figure 1). A total of 25 of the 28 strains belong to the same cluster and, as a whole, they were found to share a distant genetic relationship with most of the early isolates from China; the exceptions were Tianjin (serovar B, GenBank Accession No. AY622379), SD-1 (serovar C, GenBank Accession No. AY388647), and Dalian (serovar B, GenBank Accession No. AY622378), and the overall homology with the reference strains was at least 87.8%. Compared with the early isolates, the new sequences contained insertions and deletions. Most of the isolates (25/28) showed similar changes to those of the early isolates from China, with 3 extra bases at positions 426 to 428.

Pathogenicity Tests

Clinical Signs All 4 of the field strains induced the clinical manifestations of IC in the chickens to varying degrees. The typical signs of nasal discharge and facial swelling were observed in the infection-challenged chickens. Edema and nasal discharge were observed as mild and moderate signs in the chickens. Hematoma, temporary blindness and conjunctivitis were observed as severe signs in the chickens. Strains HeB19, HuN22 and BJ26 were responsible for 100% morbidity but the extent of the clinical signs varied among the chickens (Figure 2).

Average Scores for the Clinical Signs During this study, we plotted the scores of the clinical signs to represent the differences for each isolate over the 10-D observation period to identify any trends. The various isolates showed some differences in their virulence to chickens of 9 wk old (Figure 3). The group challenged with the HuN22 strain had higher mean disease scores (1.55) than the rest of the groups ($P < 0.05$); that is, 0.95 for HeB19, 0.82 for BJ26, and 0.32 for BJ28. No clinical signs were observed in the uninfected negative control group (Table 2).

Re-isolation Rates The bacteria re-isolated from a few chickens exposed to four field strains on 10 dpc using media formed typical satellite growth patterns on blood agar. The re-isolation rates for *Av. paragallinarum* HeB19, HuN22, BJ26, and BJ28 strains were 30, 30, 20, and 10%, respectively. Strains were not isolated from the negative control group (Table 2).

Efficacy Tests

Average Scores for the Clinical Signs The scores for the clinical signs in the infection-challenged groups are shown in Table 3. Chickens in the bivalent vaccine group challenged with the HuN22 vaccine showed similar clinical signs as those in the positive control group. The daily score and the average clinical sign score for the trivalent vaccine group and the 2 experimental vaccine groups challenged with the HuN22 strain were lower than those of the positive control group. The 2 commercial vaccine groups and the HuN22 vaccine group, which were challenged with the BJ26 strain, shared the same clinical manifestations with the positive control group. The average daily score for the BJ26 group was 0.35 points lower than that of the positive control group. None of the 4 vaccines afforded complete protection against the 2 field strains after a single vaccination. The clinical signs seen in the 4 immunization groups differed markedly (Table 3). The bivalent commercial vaccine induced no protection against the 2 isolates. While the trivalent commercial vaccine performed much better than the bivalent commercial one, the protection levels of the former were only 20 and 30%, respectively. The inactivated HuN22 vaccine had a protective effect against challenge with the HuN22 strain, with a protection rate of 40%, but no protection against the BJ26 strain. Cross protection in the groups vaccinated with the BJ26 vaccine was higher than in the groups vaccinated with HuN22 or either of the 2 commercial vaccines, and the protection levels of the chickens vaccinated with the BJ26 vaccine were 40 and 44%, respectively.

Re-isolation Rates The re-isolation rates, as based on the media from the samples obtained from the animals euthanized on 10 dpc, are depicted in Table 3. After challenge infection with the HuN22 strain, the bacterial isolation rate for the commercial vaccines was

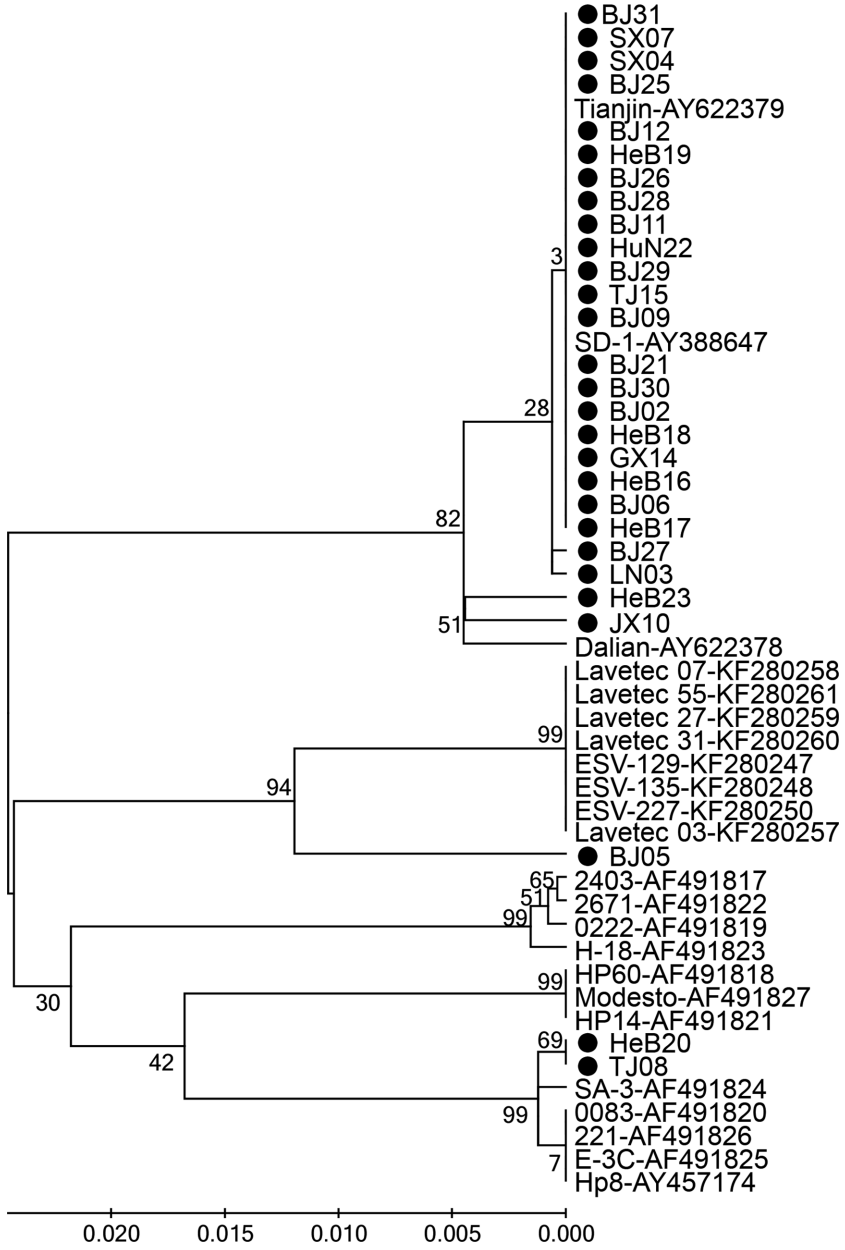

Figure 1. Phylogenetic tree showing the relationships between the *hagA* gene sequences of *Av. paragallinarum* strains. The tree was constructed using the neighbor-joining method in MEGA version 4.1. The isolates involved in this study are marked with a black dot (●).

0%. In contrast, the HuN22 vaccine group had the highest isolation rate of 40%, while the BJ26 vaccine group had an isolation rate of 30%. After challenge infection with the BJ26 strain, the bacterial isolation rate for the bivalent vaccine group was 0%, the isolation rates of the trivalent vaccine group, and the HuN22 group were 10%, while the BJ26 group was 11%. No bacterial strains were isolated from the negative control group (Table 3).

DISCUSSION

Many new or variant *Av. paragallinarum* strains have been isolated from chickens in China in recent years (Sun et al., 2018; Wang et al., 2018). During 2013 to 2018, we isolated 28 *Av. paragallinarum* strains from commercial chicken flocks displaying the typical signs of IC. Phylogenetic analysis, pathogenicity, and efficacy tests were performed to reveal the characteristics of the new variants.

HAs have been shown to be protective antigens of *Av. paragallinarum*. Nucleotide sequence analysis revealed that the HA from A9 shares 95.6% homogeneity with strain H-18 and 94.4% with strain 221. This indicates that HA is a commonly conserved antigen expressed in *Av. paragallinarum* strains, and could be a good immunogen in a subunit vaccine (Hsu et al., 2007). In the present study, HA genes were cloned and sequenced to characterize the field isolates circulating in China over recent years. Results showed that

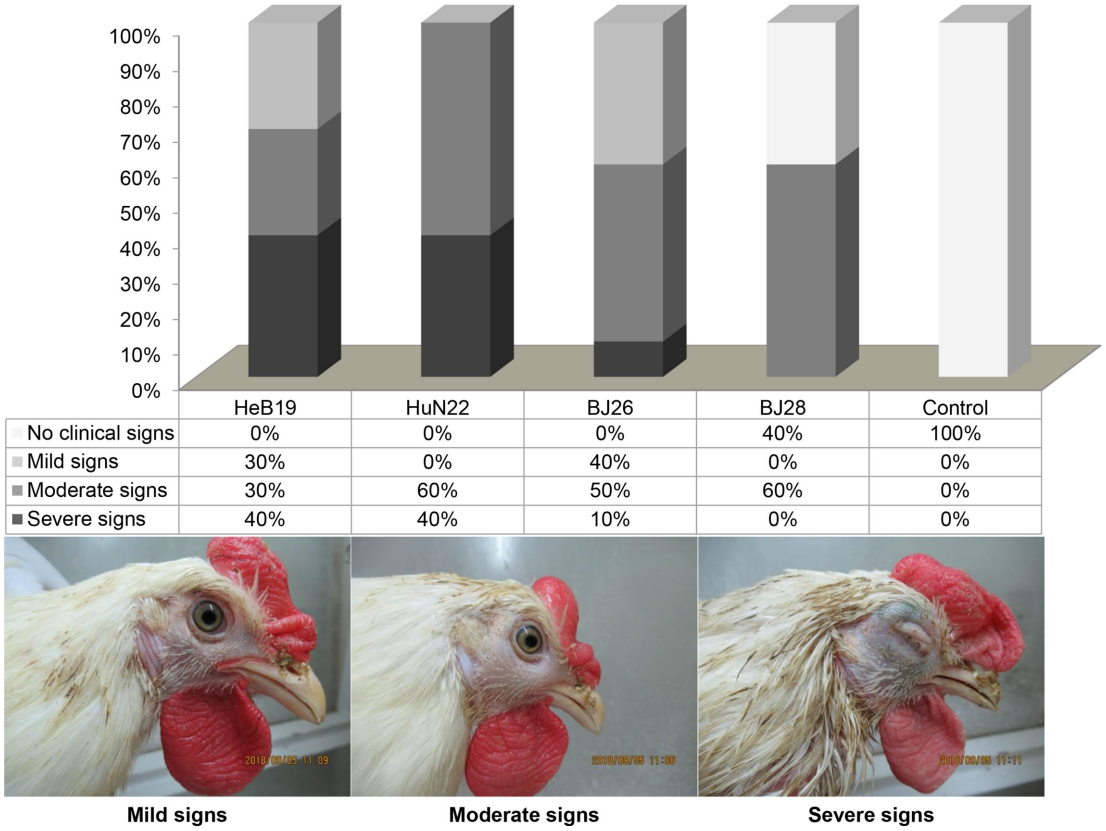

Figure 2. The proportion of mild, moderate or severe signs of chickens infected with different *Av. paragallinarum* isolates.

the prevalent *Av. paragallinarum* strains possess new characteristics and most of them shared distant genetic relationships with most of the previously characterized Chinese isolates, indicating the possible reason for the continuously occurring IC outbreaks in recent years.

The clinical manifestations of the different infection models have not been fully evaluated or standardized. Two animal experimental infection models are currently used to study virulence in *Av. paragallinarum*: the artificial intrasinus-injection-route model and the "in-contact" challenge model (Matsumoto and Yamamoto, 1975; Rimler et al., 1977; Bragg, 2002). Both models are used to evaluate bacterial pathogenicity and both have their own characteristics. In this study, we evaluated virulence in the field isolates and the protective efficacy of IC vaccines using a rapid artificial intrasinus-injection-route model. Subsequently, we observed that all 4 field isolates were virulent and able to cause IC disease in chickens. All 4 isolates formed 1 disease peak in their disease profiles during the study period. *Avibacterium paragallinarum* was isolatable from the rehabilitative birds with not much difference in the infection challenge results of the four isolates. We found that the HuN22 isolate from Hunan province displayed relatively high pathogenicity towards chickens and high re-isolation rates also, indicating that its pathogenicity may be correlated with its reproductive capacity. Virulence experiments can produce variable results when the infection routes, doses and observation periods differ. Compared with the pathogenicity of the analyzed strain, it is apparent that the HuN22 isolate from China displays strong virulence toward chickens. In the vaccine experiment, one chick had 2 disease peaks emerging after challenge with HuN22 in the BJ26 vaccine group. As reported, after IC breaks out in flocks, the rehabilitative birds are long-term carriers of the bacterium, increasing the likelihood of further repeated disease break-outs on contact with the natural environment or other disease agents (Blackall et al., 1990b).

The incidence of infection with serovar B has increased significantly in China. The laboratory examination confirmed that type B was the etiological agent in the layer farms. There is clear evidence that isolates of Page serovar B are as pathogenic as the other 2 serovars in this scheme (Zhang et al., 2003). Determining the pathogenicity of field isolates is recognized as important for predicting changes in the prevalence patterns of *Av. paragallinarum* and to avoid vaccine failure. There have been several reports in the past decade indicating that the vaccines in use were not able to provide adequate protection in different countries (Bragg et al., 1996; Terzolo et al., 1997). The lack of cross-protection among serovars with whole-cell-inactivated vaccines has probably been responsible for the emergence of variant strains and increased virulence in the isolates (Blackall et al., 1994; Soriano et al., 2004a).

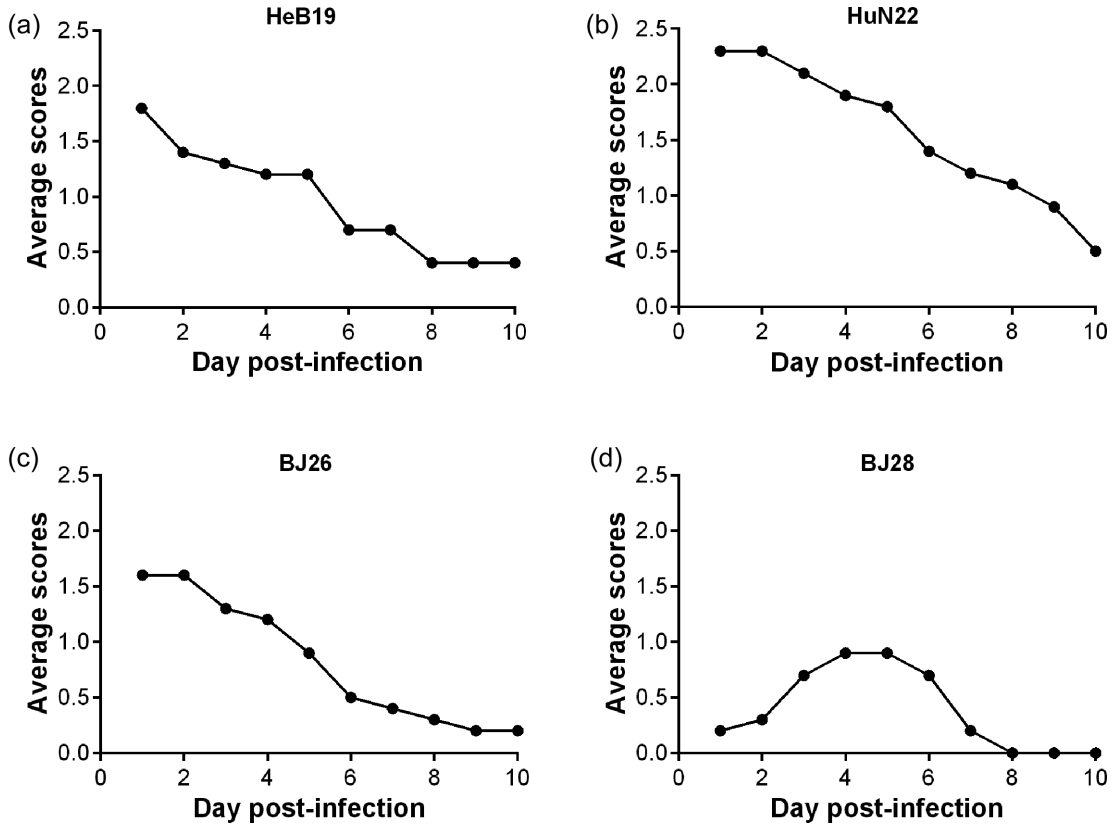

Figure 3. Average clinical scores of the 4 *Av. paragallinarum* isolates involved in this study.

Currently in China, the domestic commercial vaccine against chicken IC includes serovar A (221 strain and/or Apg-18 strain), serovar B (0222), and serovar C (H-18 strain and/or Apg-668 strain). The increased incidence of IC is related to the fact that none of the vaccines contain the correct strains, whereas the inactivated oil-emulsion vaccine containing the prevalent isolate has higher efficacy against field strains of *Av. paragallinarum*, indicating the need for a local vaccine. An experimental tetravalent oil adjuvant vaccine, containing one of the serovar B isolates, appears to be immunogenic against all the field isolates tested after one vaccination (Jacobs et al., 2003). Under the current circumstances, an oil adjuvant vaccine containing the local field isolates may be a better option for controlling the current serovar B outbreaks in China (Sun et al., 2018). In SPF chickens given a single vaccination at 42 D of age, the protection rate of the IC vaccine containing 3 isolates (one each of Page serovars A, B, and C) against all 3 serovars of *Av. paragallinarum* was at least 80% at day 30 post-vaccination (Gong et al., 2014).

In the present study, virulence in the field isolates was responsible for substantial clinical illness in the flocks. However, the 2 commercially available vaccines were not effective against the local isolates. The isolate vaccine produced better results than the commercial vaccine, indicating that the isolates might differ in their immunogenicity characteristics from the strains that are currently used in commercial vaccines. In conclusion, a trivalent or tetravalent oil adjuvant vaccine containing new field isolates may be the most useful choice for controlling IC outbreaks in China.

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