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Recombinant proteins containing the hypervariable region of the haemagglutinin protect chickens against challenge with *Avibacterium paragallinarum*

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ABSTRACT

The haemagglutinin (HA) protein plays a key role in the immunogenicity and pathogenicity of *Avibacterium paragallinarum*, but the domain organization and antigenicity exhibited by different domains of this protein remain unknown. This study reports the presence of a hypervariable region in the HA proteins of strains of serovars A and C of *A. paragallinarum*. This hypervariable region is located approximately at residues 1100–1600 of the HA protein. The sequence identity found in this hypervariable region was only 18.1%, whereas those upstream and downstream of this region were 83.8 and 97.8%, respectively. Western blot analyses using antisera against the whole-cell antigens of *A. paragallinarum* showed that the hypervariable region was more antigenic than other regions of the HA protein. Moreover, the antigenicity of the hypervariable region was serovar-specific. Chickens immunized with recombinant proteins that contained the hypervariable region were protected (83–100% protection rate) against challenge infection with *A. paragallinarum* of the homologous serovar. These results suggest that recombinant proteins containing the hypervariable region may be useful antigens for use in the development of a vaccine against *A. paragallinarum*.

1. Introduction

Avibacterium (*Haemophilus*) *paragallinarum* is the causative agent of infectious coryza, an important respiratory disease that is associated with growth retardation and reduced egg production in chickens [1,2]. Although inactivated whole-cell vaccines against *A. paragallinarum* are widely available, outbreaks of infectious coryza continue to occur and cause significant economic loss worldwide [2]. The conventional inactivated whole-cell vaccines against this pathogen have several disadvantages. First, *A. paragallinarum* has been subtyped into serovars A, B, and C according to the Page scheme [3], and inactivated whole-cell vaccines provide protection against only the serovars present in the vaccine [1]. Second, the *in vitro* growth of most strains of *A. paragallinarum* requires complex media containing nicotinamide adenine dinucleotide (NAD) [4] and some strains need chicken serum for growth [5]. The preparation of such growth media is expensive and tedious. Third, inactivated whole-cell vaccines emulsified with oil adjuvant can produce granulomas or other residual lesions after injection into chickens [6]. Thus, it is necessary to develop a new type of vaccine for the control of infectious coryza [7,8].

The haemagglutinin (HA) antigen plays a key role in the immunogenicity and pathogenicity of *A. paragallinarum*. Chickens immunized with the purified HA antigen were protected from challenge infection with *A. paragallinarum* [9]. A close correlation was found between the haemagglutination inhibition (HI) titre and protective activity [10,11]. In addition, chickens inoculated with monoclonal antibodies that possessed HI activity were protected against challenge infection with *A. paragallinarum* [12]. These results suggested that the HA is an important protective antigen for immunization against *A. paragallinarum*.

Noro et al. reported recently that the genes that encode the HA epitopes of strains of serovars A and C of *A. paragallinarum* are located at the gene fragments designated *hpa5.1* and *hpc5.5*, respectively [13,14]. Chickens that were immunized with recombinant Hpa5.1 and Hpc5.5 were protected completely against challenge infection with virulent strains of serovars A and C [13,14]. However, the molecular masses of recombinant Hpa5.1 and Hpc5.5 were extremely large (180 and 196 kDa), and these large molecular sizes led to a low level of expression and hindered the use of the two recombinant proteins as a practical vaccine antigen against *A. paragallinarum* [13,14]. We report here the identification of a

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Table 1
Primer sets used for PCR amplification of the DNA fragment from the HA gene.

DNA fragment[a]	Residues encoded[b]	Primer sets[c]
F1 (1320 bp)	71–510	5′-GGA TCC AAG TGG TTA GAG GTT TAT AGT-3′ 5′-AAG CTT ACC AAT TGC AAT GGC ATT TTT-3′
F2 (1800 bp)	71–670	5′-GGA TCC AAG TGG TTA GAG GTT TAT AGT-3′ 5′-AAG CTT CAT ATA ATC GCT ACT TTC ATT-3′
F3 (1290 bp)	425–854	5′-GGA TCC TTT AAT AGC CAT CAA ACA GGA-3′ 5′-AAG CTT TTT GTT TAA TGA ATT TTG ATC-3′
F4 (1257 bp)	640–1058	5′-GGA TCC AAA GAT ACA GAT AAT GGT ACT-3′ 5′-AAG CTT AAT TTT ACG CTC TCT CCC AGC-3′
F5 (1398 bp)[d]	1119–1584	5′-GGA TCC CAA G(C/A)T ACA ATC CAC GAT GCG-3′ 5′-AAG CTT CTT ATT CCC AGC ATC AAT ACC-3′
F6 (1884 bp)	1119–1746	5′-GGA TCC CAA GCT ACA ATC CAC GAT GCG-3′ 5′-GTC GAC TTG TGC GGC TAA TTT TGC CTT-3′
F7 (2370 bp)	1119–1908	5′-GGA TCC CAA GCT ACA ATC CAC GAT GCG-3′ 5′-GTC GAC TCC CTT TTG AGC ATC AGG ATC-3′

[a] The size of the PCR-amplified fragment is shown in parentheses.
[b] The number refers to the position of amino acid from the N-terminus of the HA protein of strain 221.
[c] The sequences of restriction enzyme (*Bam*HI, *Hin*dIII and *Sal*I) cutting sites are underlined.
[d] This primer set was used to PCR-amplify F5 from strains 221, H18 and TW07.

hypervariable region in the HA protein from strains of serovars A and C of *A. paragallinarum*. Moreover, recombinant proteins that carry sequences of this hypervariable region could be expressed in *E. coli* with high yields and chickens immunized with these recombinant proteins were protected against challenge infection with a virulent strain of *A. paragallinarum*.

2. Materials and methods

2.1. Bacterial strains and DNA extraction

Strains 221 (Page serovar A) and H18 (Page serovar C) [15], which are two reference strains of *A. paragallinarum*, together with two Taiwanese field strains, TW96 and TW07, were used in this study. TW07 was a virulent field strain. The identities of all the *A. paragallinarum* strains were confirmed by species-specific PCR [16]. TW96 and TW07 were serotyped to be Page serovar C strains as described previously [17]. All strains were grown at 37 °C on brain–heart infusion (BHI) agar supplemented with 5% bovine serum and 0.01% NAD. The plates were incubated in a candle jar at 37 °C. Bacterial DNA was isolated using the DNeasy tissue kit (Qiagen, Hilden, Germany).

2.2. "Similarity plot" analysis and Pfam search

The complete nucleotide sequences of the HA genes from strains 221 (serovar A) and KA (serovar C) have been reported previously [13,14]. The HA genes from 221 and KA have the potential to encode proteins that contain 2042 and 2039 amino acids, respectively. The level of sequence similarity between various regions of the HA proteins from 221 and KA was evaluates with a "similarity plot" analysis. This plot was constructed using software PileUp and PlotSimilarity provided by the GCG package (Genetic Computing Group, Madison, WI). The domain organization of the HA protein was investigated using a Pfam search, which predicts the functional domains of a protein on the basis of its amino acid sequence [18].

2.3. Expression of the recombinant HA proteins

Seven DNA fragments (F1–F7), covering different regions of the HA gene, were PCR-amplified with the use of the primer sets indicated in Table 1. The amplified products were digested with suitable restriction enzymes (*Bam*HI, *Hin*dIII, or *Sal*I) and then cloned into the expression vector pET28a (Novagen, Inc., Madison, WI, USA). The identity of the insert in pET28a was verified by DNA sequencing. The recombinant plasmids were transformed into *E. coli* strain BL21 (DE3), and recombinant proteins were produced as described previously [19]. In brief, *E. coli* strain BL21 (DE3) harbouring the recombinant plasmid was cultured in 50 ml of LB medium at 37 °C until the absorbance at 600 nm reached 0.6. Isopropylthio-β-D-thiogalactose (IPTG) was added to a final concentration of 0.4 mM, and the culture was grown for a further 5 h. The cells were pelleted by centrifugation at 3000 × *g* for 20 min and resuspended in 2 ml phosphate-buffered saline (PBS buffer, pH 7.0). The suspension was sonicated and centrifuged at 12,000 × *g* for 20 min. The supernatant was collected and referred to as the crude extract of *E. coli* that expressed recombinant proteins. The protein concentration was determined using a protein assay kit (BIO-RAD, Hercules, CA, USA).

2.4. Western blot

Hyperimmune sera against *A. paragallinarum* were prepared by subcutaneous immunization of 12-week-old specific-pathogen-free (SPF) chickens with 2.5×10^8 CFU (colony forming units) of *A. paragallinarum* inactivated with 0.5% formalin. Hyperimmune sera against the recombinant proteins were prepared by immunization of chickens with 200 μg of the crude extract of *E. coli* that expressed recombinant proteins in ISA-70 adjuvant (Seppic, France). A booster immunization was administered 3 weeks after the primary immunization, and sera were collected 2 weeks after the booster immunization. For each type of antigen, sera were collected from three chickens and pooled before Western blot analyses or haemagglutination inhibition (HI) tests. For Western blot analyses, the whole-cell lysates of *A. paragallinarum* or *E. coli* were subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) using the Mini-Protean 3 system (BIO-RAD). The proteins were transferred onto a nitrocellulose membrane using the semi-dry transfer system (BIO-RAD). The membrane was blocked with 3% skimmed milk and probed with murine monoclonal antibodies against hexa-histidine at 1:2000 dilution (Amersham Biosciences, Piscataway, NJ) or with chicken sera against *A. paragallinarum* or against recombinant proteins at 1:1000 dilution. The chicken sera were incubated with 250 μg/ml of the crude extract of *E. coli* BL21 (DE3) before being used to pre-absorb anti-*E. coli* antibodies. Immune complexes were detected using alkaline phosphatase-labelled anti-mouse IgG at 1:2000 dilution or anti-chicken IgG at 1:5000 dilution (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA).

2.5. Vaccination and challenge studies in SPF chickens

The SPF chickens used in this study were obtained from Animal Health Research Institute (AHRI), Council of Agriculture, Taiwan. These SPF chickens were established in AHRI and were raised in a filtered-air, positive-pressure house. Serological tests were conducted periodically in AHRI to ensure their pathogen free status. Three experiments in SPF chickens were conducted in this study. In experiments 1 and 2 the antigens were emulsified with ISA-70 whereas in experiment 3 the antigens were emulsified with a double emulsion adjuvant [20]. In each experiment, 5-week-old SPF chickens were randomly assigned to 4 groups; each group contained 6 chickens. The chickens in groups 1, 2 and 3 were immunized subcutaneously with 200 μg of the crude extract of *E. coli* that expressed recombinant HA proteins derived from strains TW07, H18 and 221, respectively. The chickens in group 4 (the control group) were immunized with 200 μg of the crude extract of *E. coli* host strain. Three weeks after the primary immunization, a booster immunization was conducted, and 3 weeks after booster immunization, the chickens were challenged with intranasal inoculation of 5×10^9 CFU of a virulent field strain TW07. The vaccination and challenge schedule used in this study was adopted from previous studies [13,14,21,22]. The strain TW07 was used as the challenge strain because it produced more severe clinical signs (nasal discharge and facial edema) than other strains we had. The chickens were examined for clinical signs on days 1–7 after the challenge. This examination period was according to previous studies [13,14,22]. On day 7 after the challenge, infraorbital sinus swabs from all chickens were streaked onto a BHI agar plate supplemented with 5% bovine sera and 0.01% NAD. The re-isolated *A. paragallinarum* were confirmed by PCR [16]. A protected chicken was defined as one that showed no clinical signs during the period of observation and failed to produce *A. paragallinarum* upon culture from the sinuses. Differences in protection rates were compared by chi-squared tests using SAS software (SAS Institute Inc., Cary, NC, USA). The animal experiments were performed according to current regulations and approved by the Institutional Animal Care and Use Committee (IACUC) at National Chung Hsing University.

2.6. Haemagglutination and haemagglutination-inhibition (HI) tests

The HA antigen of strain 221 was prepared from untreated cells, whereas those of strains H18 and TW07 were prepared from enzyme-treated bacterial cells [17]. In brief, bacterial cells grown at 37 °C for 20 h on a BHI agar plate were collected and resuspended in 2 ml of phosphate-buffered saline (PBS, pH 7.0). The cells were washed three times with PBS, and the number of cells in PBS was adjusted to 2×10^{11} CFU/ml. The H18 and TW07 cells were incubated with bovine testicular hyaluronidase (5 U/ml, Sigma–Aldrich) and *Flavobacterium heparium* heparinase III (0.5 U/ml, Sigma–Aldrich), respectively, at 37 °C for 2 h. The enzyme-treated cells were washed three times with PBS and resuspended in the original volume of PBS. The haemagglutination tests were conducted using 1% (v/v) glutaraldehyde-fixed chicken erythrocytes [23].

For the HI tests, chicken antisera were preabsorbed with a four-fold volume of 10% (v/v) glutaraldehyde-fixed chicken erythrocytes containing 500 μg/ml of the crude extract of *E. coli* BL21 (DE3) to remove nonspecific reactions. The preabsorption was conducted at 37 °C for 2 h with shaking, followed by centrifugation at 10,000 × *g* for 20 min. The supernatants were collected and referred to as the five-fold diluted absorbed antisera, from which a series of two-fold dilutions from 1:5 to 1:640 were made using PBS. The diluted sera (50 μl) were mixed with an equal volume of antigen containing 4 haemagglutination units and incubated at room temperature for 15 min. Then, 50 μl of 1% (v/v) glutaraldehyde-fixed chicken erythrocytes were added and the HI titres were determined after 1 h at room temperature.

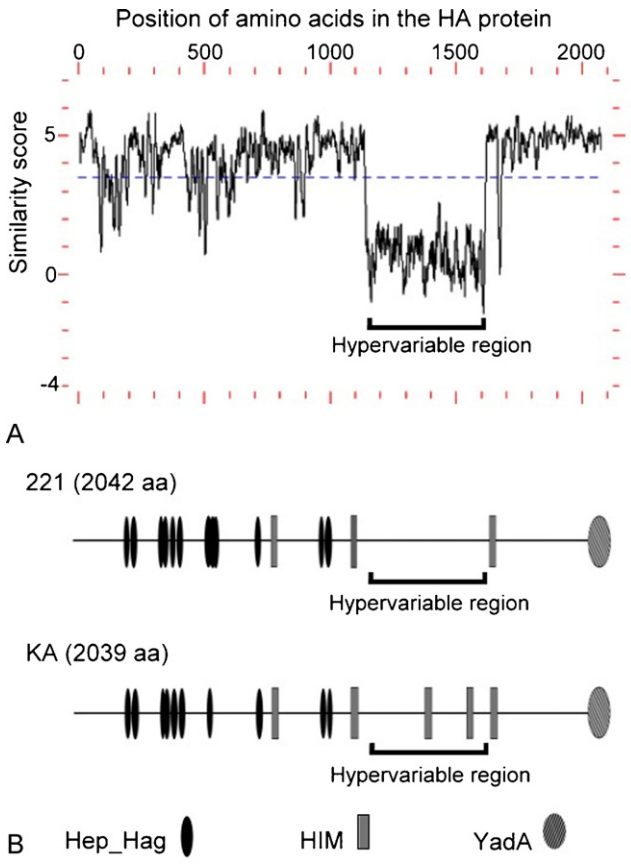

Fig. 1. (A) Similarity plot of sequences of the HA proteins from strains 221 (serovar A, accession numbers AR303123 and DD214059) and KA (serovar C, accession numbers AR303126 and DL489152). The similarity score is shown at the left of the figure; a higher score indicates greater similarity. The region with hypervariable sequences is indicated by a bracket. (B) Domain organization of the HA proteins from strains 221 and KA. The hypervariable region is indicated by a bracket.

3. Results

3.1. The HA protein of A. paragallinarum contains a hypervariable region

The level of sequence similarity between various regions of the HA proteins from 221 (serovar A) and KA (serovar C) was evaluated with a "similarity plot" analysis. The result showed that a hypervariable region was present at approximately residues 1100–1600 of the HA protein (Fig. 1A). The sequence identity found in this hypervariable region was only 18.1%, whereas those upstream and downstream of this region were 83.8 and 97.8%, respectively.

The domain organization and possible function of the hypervariable region of the HA protein was investigated using a Pfam search. The result showed that the HA proteins from 221 and KA contained multiple copies of the Hep_Hag domain (PF05658) and the HIM domain (PF05662), and a single copy of the C-terminal YadA domain (PF03895) (Fig. 1B). Two HIM domains were found in the hypervariable region of strain KA, whereas no such domain was found in that of 221 (Fig. 1B). It is noteworthy that the hypervariable regions from both 221 and KA were bracketed by two HIM domains (Fig. 1B).

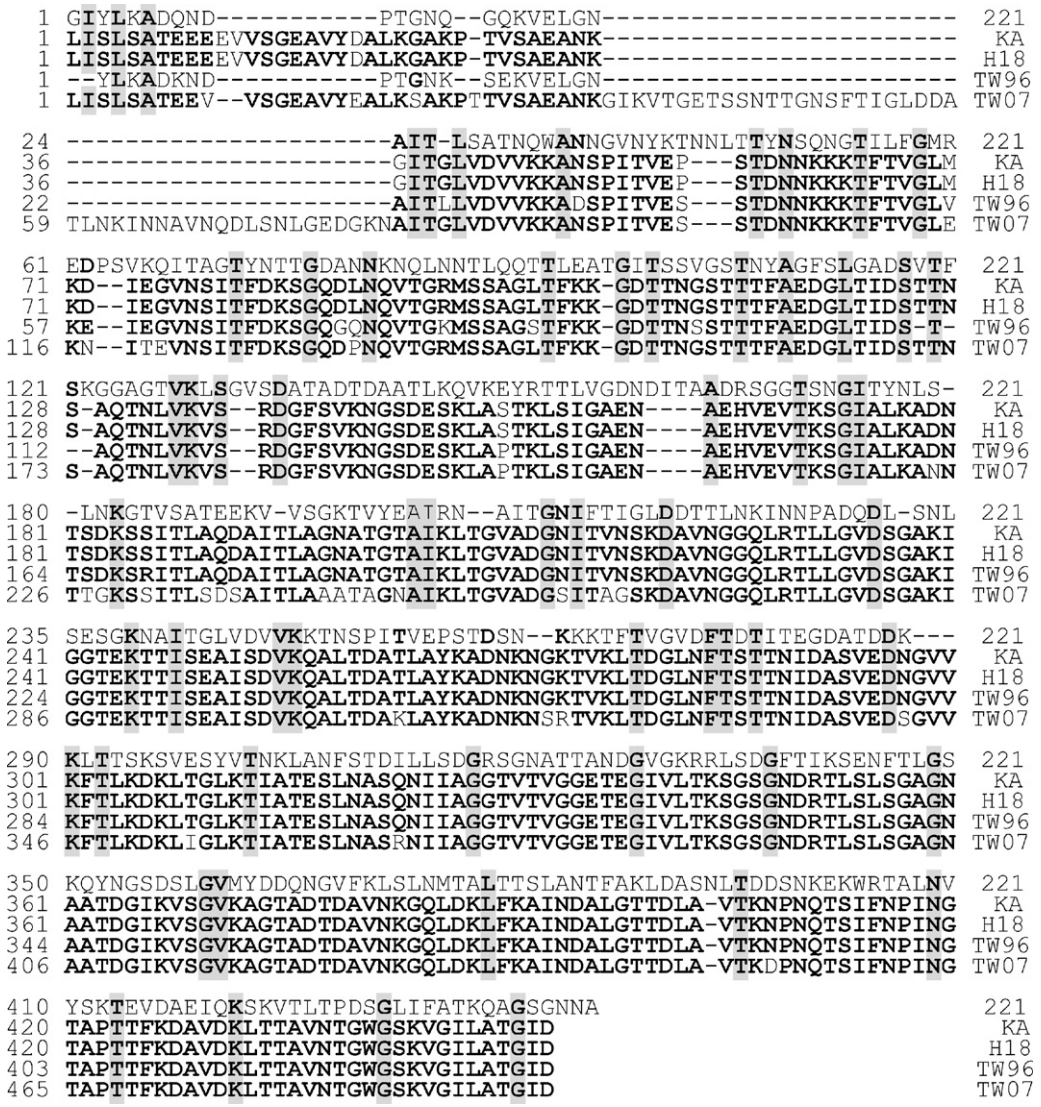

Fig. 2. Amino acid sequence alignment of the hypervariable regions from different strains of *A. paragallinarum*. The sequences aligned correspond to residues 1133–1577 of the HA protein from strain 221 or residues 1136–1587 of the HA protein from strain KA. The identical residues are in bold type and residues conserved in all strains are shadowed. This alignment was conducted using the MegAlign program in the LASERGENE package (DNASTAR Inc., Madison, WI). The nucleotide sequences determined in this study are available in GenBank under the accession numbers HM535642–HM535644.

3.2. The hypervariable region contains amino acid substitutions, insertions and deletions

To investigate whether or not the hypervariable region was present in the HA proteins of strains other than 221 and KA, we conducted sequence analysis of the HA genes from the reference strain H18 (serovar C) and two Taiwanese field strains, TW96 and TW07 (both are serovar C) [17]. The result showed that the hypervariable region was present in these three strains. The sequences of the hypervariable region from strains H18, TW96 and TW07 had 100, 92.5 and 84.3% sequence identity, respectively, to that of strain KA, but had only 17.5, 19.1 and 17.1% sequence identity, respectively, to that of strain 221. This result indicated that the hypervariable regions from the four strains of serovar C were similar to each other but differed substantially from that of strain 221. The alignment of amino acid sequences of the hypervariable region is shown in Fig. 2. It can be seen that the sequence from 221 could only be aligned poorly with those from the serovar C strains (KA, H18, TW96 and TW07). In contrast, sequences of the four serovar C strains were aligned with high sequence identity, although some insertions, deletions and amino acid substitutions were found among these four strains (Fig. 2). Note that an insertion of 46 amino acids was found in the hypervariable region of the HA of strain TW07; this insertion started at residue 35 of the hypervariable region of this strain (Fig. 2).

3.3. Antigenicity of the hypervariable region

Seven DNA fragments (F1–F7), which spanned different regions of the HA gene of strain 221, were PCR-amplified and then expressed in *E. coli* as recombinant proteins (Fig. 3A). These recombinant proteins contained a hexa-histidine tag attached to their carboxyl terminus. The predicted molecular masses of the recombinant proteins of F1–F7, designated rF1–rF7, were 50.3, 66.7, 50.3, 49.5, 54.4, 70.9 and 88.3 kDa, respectively. All of these recombinant proteins could be expressed in *E. coli* (Fig. 3B). Western blot analyses using a monoclonal antibody against hexa-histidine showed that this antibody reacted with rF1–rF7 (Fig. 3C), which indicated that these recombinant proteins contained the hexa-histidine tag. Western blot analyses using chicken antisera directed against the whole-cell antigen of strain 221 showed that these sera reacted strongly with rF5, rF6 and rF7, but poorly with rF1–rF4 (Fig. 3D).

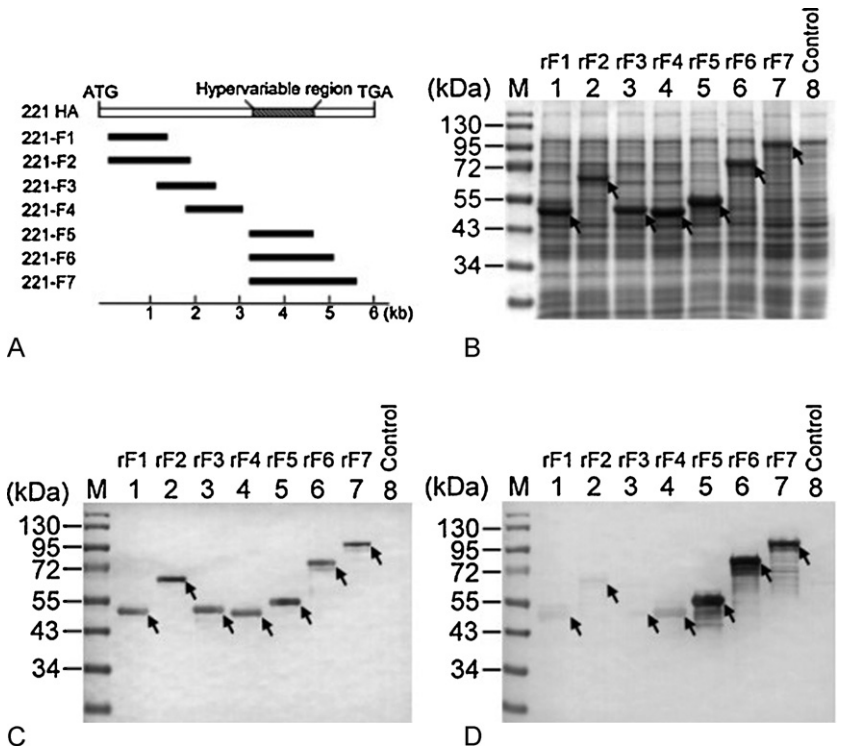

Fig. 3. (A) Location of fragments F1–F7 of the HA gene from strain 221. (B) Coomassie blue-stained SDS-PAGE of proteins from whole-cell lysates of *E. coli* that expressed rF1–rF7. Lane M represents the molecular mass markers. (C and D) Immunoblots of duplicate gels probed with murine monoclonal antibodies against hexa-histidine and chicken antisera against strain 221, respectively. The bands corresponding to rF1–rF7 are indicated by arrows.

Because rF5, rF6 and rF7, but not rF1–rF4, contain the hypervariable region, the result shown in Fig. 3D indicates that the hypervariable region is more antigenic than other regions.

To investigate whether or not the antigenicity exhibited by the hypervariable region is serovar-specific, rF5 proteins derived from strains H18 and TW07, in addition to strain 221, were expressed in *E. coli* and then used as antigens in Western blot analyses (Fig. 4). The predicted molecular masses of rF5 derived from H18, TW07 and 221 were 53.6, 58.3 and 54.4 kDa, respectively. The molecular mass of rF5 derived from TW07 was larger than that from 221 and H18, owing to the insertion of 46 amino acids in the hypervariable region of TW07. These three recombinant proteins could be expressed in *E. coli* with high yields, as judged by the strong band intensity of rF5 shown on the gel (Fig. 4A). The results of the Western blot analyses showed that chicken antisera against whole-cell antigens of strain 221 reacted with rF5 derived from 221 but not with rF5 from H18 and TW07 (Fig. 4B). In contrast, chicken antisera against whole-cell antigens of H18 and TW07 reacted with rF5 derived from H18 and TW07 but not with rF5 from 221 (Fig. 4C and D). These results indicate that the antigenicity exhibited by rF5 is serovar specific.

This serovar-specific antigenicity was further investigated by Western blot analysis with the use of the whole-cell lysates of strains 221, H18 and TW07 as the antigens, and chicken antisera against rF5 as the antisera (Fig. 5). The result showed that antisera against rF5 from 221 reacted with two protein bands of about 200 and 155 kDa from strain 221 but did not react with any cellular protein from H18 and TW07 (Fig. 5B). On the other hand, antisera against rF5 from H18 and TW07 reacted with two major protein bands of about 190 and 155 kDa from H18 and two protein bands of about 160 and 155 kDa from TW07 but did not react with any cellular protein from 221 (Fig. 5C and D). These results suggest that these reactive protein bands were derived from the HA protein of *A. paragallinarum* and that the antibodies elicited by rF5 were serovar-specific.

3.4. Immunogenicity of the hypervariable region

Chickens were immunized with a crude extract of *E. coli* that expressed rF5 derived from 221, H18 and TW07 and were then challenged with TW07. The crude extract was used because rF5 formed insoluble aggregates with the resin we used to purify this protein, and this behavior hampered the purification of rF5. Three vaccination and challenge experiments were conducted and the results were found to be very similar (Table 2). Sera collected from chickens immunized with the rF5 derived from TW07 had HI antibodies against TW07, but not against H18 or 221. In contrast, sera from chickens immunized with the rF5 derived from H18 had HI antibodies against H18 and TW07, but not against 221. Sera from chickens immunized with the rF5 derived from 221 had HI antibodies against 221, but not against H18 and TW07 (Table 2). After challenge infection with TW07, chickens immunized with the rF5 proteins derived from TW07 and H18 were protected (83–100% protection rates). These rates of protection were significantly higher than that the control group ($p < 0.05$). In contrast, chickens immunized with the rF5 derived from 221 showed protection rates of 17–33%, and these rates of protection were not significantly higher than that in the control group ($p > 0.05$) (Table 2). In summary, chickens immunized with the rF5 proteins derived from TW07 and H18 were protected against challenge infection with TW07, whereas those immunized with the rF5 from 221 were not. Therefore, the protection conferred by immunization with rF5 was serovar specific.

4. Discussion

The data presented in this study show that a hypervariable region is present in the HA protein of *A. paragallinarum*. Moreover, recombinant proteins that contain this hypervariable region conferred serovar-specific protection against challenge infection of chickens with *A. paragallinarum*. To the best of our knowledge, this

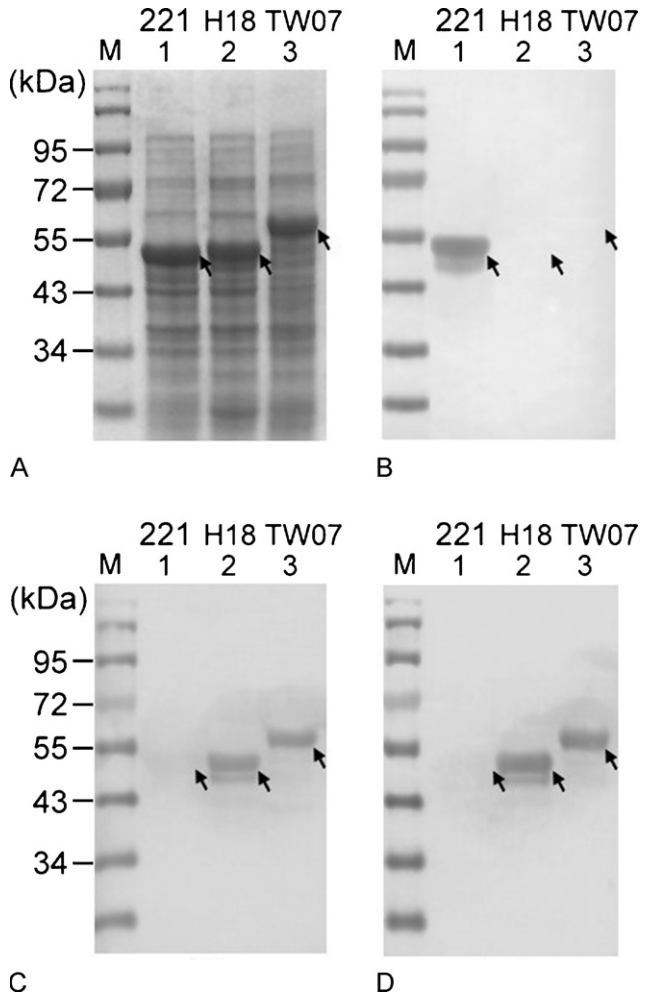

Fig. 4. (A) Coomassie blue-stained SDS-PAGE of proteins from whole-cell lysates of *E. coli* that expressed rF5 derived from 221, H18 and TW07. (B–D) Immunoblots of duplicate gels probed with chicken antisera against strains 221, H18 and TW07, respectively. The bands corresponding to rF5 are indicated by arrows.

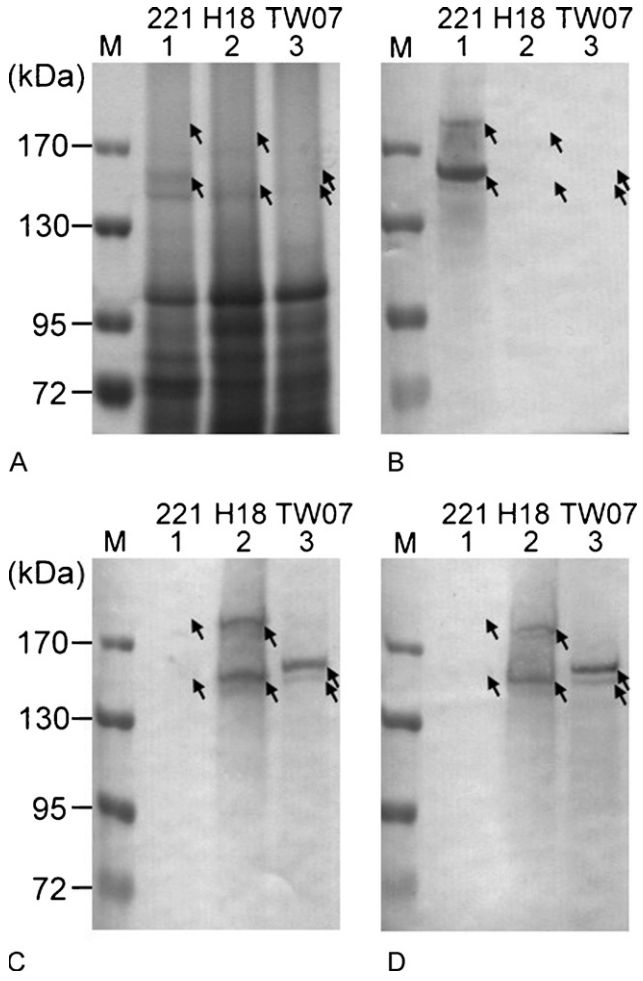

Fig. 5. (A) Coomassie blue-stained SDS-PAGE of proteins from whole-cell lysates of strains 221, H18 and TW07. (B–D) Immunoblots probed with chicken antisera against rF5 derived from 221, H18 and TW07, respectively. The bands corresponding to HA proteins or their processed products are indicated by arrows.

is the first report of the presence of a hypervariable region in the HA protein of *A. paragallinarum*, and it demonstrated the protective efficacy conferred by immunization with proteins containing this region. Although it has been reported previously that immunization of chickens with recombinant HA proteins (Hpa5.1 and Hpc5.5) conferred protection, the large molecular mass of Hpa5.1 and Hpc5.5 led to a low level of expression and hindered their use as a vaccine antigen in practice [13,14]. In contrast to Hpa5.1 and

Table 2
Results of immunization and challenge tests in SPF chickens.

Immunized with	HI titre against[a]			Protection rate (%) against TW07[b]
	TW07	H18	221	
Experiment 1				
TW07-rF5	168.3	<5	<5	100 (6/6)*
H18-rF5	100.0	46.7	<5	83 (5/6)*
221-rF5	<5	<5	33.8	17 (1/6)
Control	<5	<5	<5	0 (0/6)
Experiment 2				
TW07-rF5	143.3	<5	<5	100 (6/6)*
H18-rF5	116.7	38.4	<5	100 (6/6)*
221-rF5	<5	<5	36.7	17 (1/6)
Control	<5	<5	<5	17 (1/6)
Experiment 3				
TW07-rF5	246.7	<5	<5	100 (6/6)*
H18-rF5	125.0	43.8	<5	83 (5/6)*
221-rF5	<5	<5	25.0	33 (2/6)
Control	<5	<5	<5	0 (0/6)

[a] The HI titre was the reciprocal of highest dilution of antiserum showing inhibition of haemagglutination titre and each titre was the geometric mean of sera collected from 6 chickens.
[b] The asterisk (*) indicates the rate differs significantly ($p < 0.05$) from the control group.

Hpc5.5, recombinant proteins containing the hypervariable region were only 53.6–58.3 kDa in size and could be produced with high yields in *E. coli*. Therefore, these recombinant proteins may serve as useful antigens for protection against *A. paragallinarum*. Given that the costs for culturing *E. coli* and using it to produce recombinant proteins are generally lower than those involved in the production of *A. paragallinarum*, application of these recombinant proteins in vaccines is expected to contribute to the control of infectious coryza in the field.

The result of Western blot analyses showed that the antigenicity of rF5 was serovar specific. In contrast, the results of HI tests showed that the HI antibodies elicited by rF5 were either strain or serovar specific. Antisera against the rF5 derived from 221 and TW07 had HI activity against only 221 and TW07, respectively, whereas antisera against the rF5 derived from H18 had HI activity against both H18 and TW07, but not against 221. The lack of HI reactivity against H18 in antisera against the rF5 derived from TW07 was unexpected, because both H18 and TW07 are strains of serovar C. This observation suggests that, in addition to the hypervariable region, other regions in the HA protein of H18 may play a role in eliciting HI antibodies. Further investigation is required to address this issue.

The result of the Pfam search showed that the HA protein contains multiple Hep_Hag and HIM domains together with a single YadA domain. The Hep_Hag and HIM domains are found in many bacterial haemagglutinins and adhesins [24], while the C-terminal YadA domain has been found in a family of bacterial proteins known as trimeric autotransporter adhesins (TAAs) [25]. These results suggest that the HA protein of *A. paragallinarum* is a member of the TAAs family [25,26]. Proteins of the TAAs family have a general structure that consists of head, neck, stalk and anchor domains. In *Yersinia* adhesion A, the best-characterized TAA, the head region is comprised of multiple Hep_Hag domains, the neck region by a HIM domain, and the anchor region by a YadA domain [27]. Given that the HA protein of *A. paragallinarum* contains 10–12 copies of the Hep_Hag domains and 3–5 copies of the HIM domains, it appears that the HA protein has multiple head and neck regions. It remains to be determined how these multiple head and neck regions may contribute to the haemagglutination activity of the HA protein and to the binding of *A. paragallinarum* to the surface of the host cells.

The function of the hypervariable region remains unclear. Given that this region contains no Hep_Hag domain and is bracketed by two HIM domains, it is possible that the hypervariable region is part of the stalk region. Two major functions are assigned to the stalk region of TAAs: the first is to project the head region from the bacterial cell surface and the second is to protect the bacteria against host defences by conferring serum resistance [28,29]. The exact mechanism of serum resistance mediated by the stalk region remains unclear. It is possible that the binding of the stalk region to the complement inhibitor factors C4BP, C3 and vitronectin may be involved in this process [30–32]. In the HA protein from *A. paragallinarum*, the stalk region appears to contain a hypervariable sequence, and it will be intriguing to investigate whether this stalk region has the ability to confer serum resistance and whether the sequence diversity found in this region plays a role in this process.

The mechanism by which the hypervariable region was generated remains unknown. In other TAAs, recombination via the repeated sequences is important in generating the sequence diversity of TAAs, and this diversity is related to the adaption of a bacterium to its host [25,33]. Given that the hypervariable region of the HA of *A. paragallinarum* is bracketed by two HIM domains, we propose that the hypervariable sequence originated from homologous recombination via the HIM domains.

The results of Western blot analyses showed that the molecular masses of the HA proteins of *A. paragallinarum* were about 155–200 kDa. These molecular masses were lower than those (211–213 kDa) predicated from the nucleotide sequences of the HA genes [13,14]. This discrepancy may have resulted from posttranslational processing of the HA protein. Such processing has been reported for the filamentous haemagglutinins from *Bordetella pertussis* and *Pasteurella multocida* [34,35].

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