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# Sequence analysis of the hypervariable region in *hmtp210* of *Avibacterium paragallinarum*

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**ABSTRACT.** The *hmtp210* gene of *Avibacterium paragallinarum*, the causative agent of infectious coryza, encodes an outer-membrane hemagglutinin (HA) that plays an essential role in pathogenicity. A hypervariable region within this HA, which is highly antigenic, is proposed as a candidate for recombinant vaccine production. Nonetheless, little is known about its genetic variability. We performed sequencing analysis of the *hmtp210* hypervariable region in 16 clinical isolates from Costa Rica and compared them with 4 vaccine strains and the *hmtp210* sequences available in public databases. Except for isolate ApCR12, all isolates showed high identity with reference vaccine strains 0083 and H18. Better genetic characterization of the hypervariable region of *hmtp210* is necessary to develop better immunogenic strategies and improved molecular typing methods.

**KEY WORDS:** *Avibacterium paragallinarum*, infectious coryza, Multiplex PCR, nucleotide sequencing, PCR-RFLP

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Bacterium *Avibacterium paragallinarum* is the causative agent of infectious coryza. This is an acute respiratory disease of economic significance in poultry and leads to a decrease in egg production and failure or retardation of egg laying [1]. Serological classification of *A. paragallinarum* is based on the presence of specific hemagglutinins (HAs). *A. paragallinarum* strains/isolates are traditionally serotyped by means of 2 schemes: the Page scheme, which recognizes 3 serovars, A, B and C [3, 13], and the Kume scheme, which recognizes serogroups A, B and C and 4 serovars within Kume serogroup A, 1 serovar within Kume serogroup B, and 4 serovars within Kume serogroup C [4, 7]. Recently, a multiplex PCR and PCR-RFLP method for serotyping of *A. paragallinarum* was proposed [15]. Recent studies have shown that these molecular methods have poor sensitivity and specificity and are therefore not suitable for serotyping *A. paragallinarum* [11, 19].

Prevention of infectious coryza has been primarily implemented by immunizing animals with inactivated whole-cell vaccines of *A. paragallinarum* prepared from relevant serovars. Whole-cell vaccines of *A. paragallinarum* protect only from the HA serovars present in them. If chickens are challenged with a serovar not included in the vaccine that was used, then this vaccine will not provide adequate protection [2]. Additionally, such vaccines occasionally have side effects such as local necrotic lesions and swelling at the injection site [10].

Gene *hmtp210* of *A. paragallinarum* encodes a 210-kDa outer-membrane protein that functions as an HA [18] and has been identified as an important protective antigen [12]. It has also been proposed that the HA antigen plays a key role in the pathogenicity of *A. paragallinarum* [20]. Wang *et al*. [20] showed that HA is a trimeric autotransporter adhesin that besides conferring hemagglutination, enables cell adherence and biofilm formation activities.

Wu *et al*. [21] reported the presence of a hypervariable region in the HA proteins in isolates of Page serovars A and C. This hypervariable region is located approximately at amino acid positions 1,100–1,600 of the HA protein. Hypervariable regions of serovars A and C (also known as region 2) are said to be the most antigenic region of the HA protein [16, 21]. Consequently, this protein has been reported as a candidate for serotyping and recombinant-vaccine production [15, 21]. Sakamoto *et al.* [16]

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published the development of recombinant vaccines from *hmtp210* based on the variability of region 2. Using Sakamoto's PCR methods [15], Wang *et al.* [19] performed sequence analysis of the multiplex PCR products and demonstrated that this region 2 is not Page serovar specific.

In spite of the importance of this hypervariable region in terms of pathogenicity and protective properties, little is known about its genetic diversity, variability and complexity. This is in part because only a small number of *hmtp*210 variants have been sequenced and uploaded to public databases, especially from Latin America.

To resolve this issue, we performed sequencing analysis of the hypervariable region of *hmtp210* in 16 clinical isolates from the largest poultry production area of Costa Rica and compared them with the data from 4 vaccine strains and all *hmtp210* sequences available in public databases.

Animals with clinical signs of infectious coryza were subjected to necropsy in the Avian Pathology Department at the Veterinary Medicine School, Universidad Nacional, and samples were collected from infraorbital sinuses. Bacterial strains/isolates were cultured in Chocolate Agar supplemented with Vitox (Oxoid, Hampshire, England) and biochemically identified according to protocols described elsewhere [9]. A total of 16 field isolates (Table S1) and 4 vaccine strains—0083 (Kume serovar A-1), Spross (Page serovar B), 48 (serotype variant type B) and H18 (Kume serovar C-1)—were subjected to further genetic analysis.

For genomic-DNA extraction, a loopful of bacteria was resuspended in 50 $\mu l$ of buffer EB (10 mM Tris-Cl, pH 8.5). The suspension was heated at 98°C for 15 min and placed on ice for additional 15 min. The samples were stored at −20°C until used. A species-specific polymerase chain reaction (PCR) for *A. paragallinarum* was carried out by means of primers and amplification steps previously described [5]. PCRs were conducted in a total volume of 25 $\mu l$ containing *Taq* DNA Polymerase, $MgCl_2$ buffer, and dNTPs according to the PCR Master Mix Kit (Promega, Madison, WI, U.S.A.), 0.2 $\mu$M of each specific primer and 3 $\mu l$ of extracted DNA (50 *n*g/m*l*). Genetic protocols (Multiplex PCR and restriction fragment length polymorphism [RFLP] PCR) and reaction conditions were previously described [15]. RFLP PCR products (including the entire length of the HA hypervariable region) were digested with the *Bgl* II restriction enzyme (Fermentas, Glen Burnie, MD, U.S.A.). RFLP PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germantown, MD, U.S.A.), and Sanger sequencing was performed at Macrogen (Seoul, Korea) with the following primers: 5–1 forward, 5′-GAT GGC ACA ATT ACA TTT ACA-3′, and 5–1 reverse, 5′-ACC TTG AGT GCT AGA TGC TGT AGG TGC-3′ [15]. The obtained sequences were edited using Sequencher® version 5.3 sequence analysis software.

To construct a multiple sequence alignment for phylogenetic reconstruction, sequence data from 76 *A. paragallinarum* strains/isolates (16 Costa Rican field strains, 4 vaccine reference strains, and full-length *hmtp210* sequences from 56 strains/isolates available at the National Center for Biotechnology Information [NCBI] GenBank) (Table S2) were visualized, similarly edited, aligned, and analyzed in MEGA version 7 [6]. The resulting alignment of 1,068 positions was used to build a phylogenetic tree by the maximum likelihood method based on the Tamura-Nei model [17]. The tree with the highest log likelihood was selected. All the positions containing gaps or missing data were eliminated. Initial trees for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then by selecting the topology with a superior log likelihood value.

In addition, representative isolates that clustered together or closely (in the phylogenetic tree) to reference strains 0083, Spross, and H18, were aligned for comparison. The variable sites in the final alignment from each group were extracted by means of SAMtools [8]. The generated vcf/bcf files were used to produce a visual representation of the variants in the alignment. The variants were colored following the standard color scheme used in Artemis software [14].

After biochemical and PCR testing, 16 *A. paragallinarum* field isolates were identified (Table S1). According to Sakamoto's typing methods [15], most of the Costa Rican isolates corresponded to Page serovar C (14 strains). Only 1 isolate corresponded to Page serovar A and 1 to Page serovar B (Fig. S1). In contradiction to Wang's report [19], our H18 reference strain (KY819140) was correctly identified by Sakamoto's typing methods as Page serovar C (Fig. S1).

Subsequently, full-length sequencing of the hypervariable region of *hmtp210* in the 16 field strains and 4 reference vaccine strains was conducted. These 20 sequences were aligned with 56 sequences available in GenBank, and phylogenetic analysis was carried out. As shown in Fig. 1, several clusters formed. Strains/isolates that belonged to the same Page serovar, did not necessarily gather in the same cluster. This result is consistent with the observations made by Wang *et al*. [19], who proposed that this sequence is not completely Page serovar specific. With the exception of 4 strains/isolates, serovar C strains and isolates (including 14 isolates from Costa Rica and 2 H18 strains) remained in the same cluster, were highly conserved, and showed short distances when compared to the H18 strain we used. Moreover, all Costa Rican isolates from this cluster had clonal relations and shared 99.8% identity with our H18 strain. This finding is remarkable because these samples were collected at different time points, and most of them came from different farms. It is also worth noting that one of the most divergent serovar C strains was also an H18 strain (KU143741). This result casts doubt on the accuracy of this sequence or the validity of this reference strain.

Several Page serovar A strains/isolates (including our 0083 strain and ApCR16 isolate) formed a cluster distant from the rest of the strains, indicating substantial divergence within this *hmtp210* gene. In the center of the phylogenetic tree, many clusters with short distances among them were formed. These clusters included all Page serovars B (including our Spross and 48 strains and ApCR12 isolate) but also some strains/isolates of serovars A and C.

To take a closer look at the isolates that showed high identity with reference strains 0083, Spross, 48 or H18, the selected sequences were aligned and compared with the references. When selected serovar A isolates were compared with strain 0083, isolate VRDC/Avpg/SZ/H10 showed 4 SNPs (C-T at position 168, A-G at position 303, A-C at position 664 and A-C at position 666) and a 13-bp deletion. Isolate Gd2 showed 3 SNPs (G-A at position 251, C-A at position 255 and A-G at position 303). The

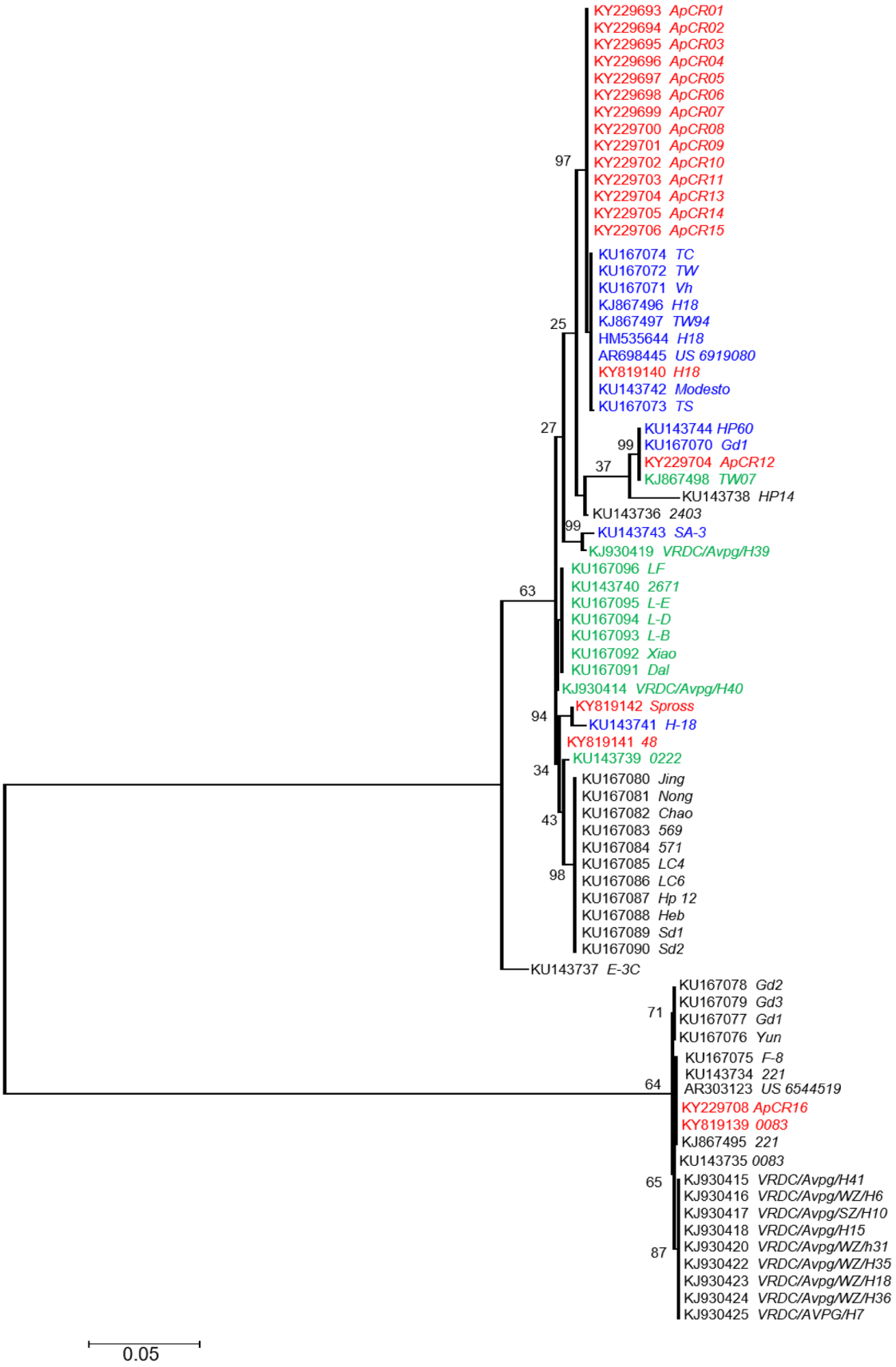


**Fig. 1.** A phylogenetic tree reconstructed from partial sequences of *hmtp210*. The tree with the highest log likelihood (−3571.2125) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were 1,068 characters in the final dataset. Black text represents serovars A. Green text represents serovars B. Blue text represents serovars C. The sequences reported in this study are red. The names of the strains/isolates are italic.

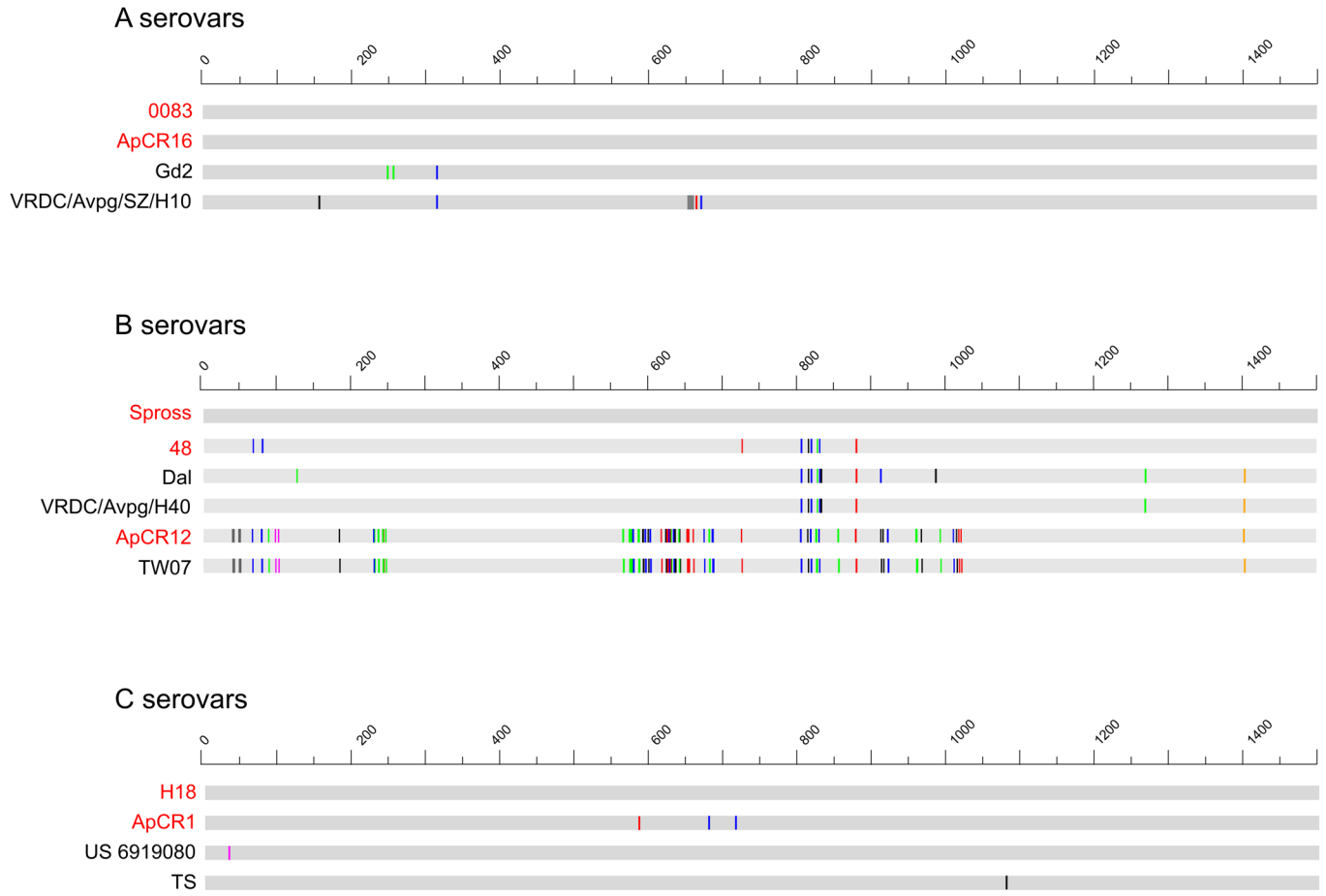


**Fig. 2.** Graphic representation of the alignment variants in representative sequences. Each group of selected sequences was aligned against reference strains 0083 (Kume serovar A-1), Spross (Page serovar B), and H18 (Kume serovar C-1). The upper line indicates approximate coordinates of the alignment in base pairs. The color scheme is as follows: variant A=green, variant G=blue, variant T=black, variant C=red, heterozygous variant=orange, insertion=magenta, deletion=dark grey, nonvariant=light grey. The reference sequences show no polymorphism. The sequences reported in this study are in red.

sequence of our ApCR16 isolate was identical to strain 0083 sequence (Fig. 2).

In the case of selected serovar B isolates, significant variations were observed when compared against reference strain Spross. Isolate ApCR12 and isolate TW07 were similar among them, but showed up to 60 SNPs, 2 deletions, and 2 insertions in comparison with strain Spross. The other 3 sequences (48, Dal, and VRDC/Avpg/H40) were found to be highly similar to strain Spross, showing 10 or fewer SNPs (Fig. 2). When selected serovar C isolates and 1 strain were compared with reference strain H18, we detected a 3-bp insertion (AGA) in the sequence at positions 40–42 in strain US 6919080, 1 SNP (C-T at position 1086) in isolate TS, and 3 SNPs in isolate ApCR1 (A-C at position 575, T-G at position 669 and C-G at position 709); these data represent identity of 99.8% with strain H18 (Fig. 2). With the exception of the ApCR12 isolate, all the Costa Rican isolates had a high percentage of identity with reference vaccine strains 0083 and H18.

When nucleotide regions were translated into amino acid sequences for comparison with the reference strains, the same tendency was observed. Costa Rican A (ApCR16) and C (ApCR1) isolates had only 1 and 2 substitutions as compared to strains 0083 and H18, respectively (Fig. S2). The ApCR12 isolate carried 3 insertions, 1 deletion, and 28 substitutions when compared to reference strain 48.

Due to a lack of sequence data on *A. paragallinarum* strains from different parts of the world, this current study may serve as a preliminary approach to understanding the genetic variability and diversity of the hypervariable region of *hmtp210*. Better genetic characterization of the hypervariable region in *hmtp210* is necessary to develop better immunogenic strategies and eventually improved molecular typing methods.

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