NOTE *Avian Pathology*

Development of a Multiplex PCR and PCR-RFLP Method for Serotyping of *Avibacterium paragallinarum*

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ABSTRACT. *Avibacterium* (*Haemophilus*) *paragallinarum* (*A. paragallinarum*) is a causative agent of infectious coryza in chickens and is classified into three serovars by agglutination tests. In an effort to identify the serovars easily, PCR and PCR-RFLP were employed. As the target gene for PCR, the hypervariable region of *HMTp210*, which encodes the HA antigen, was used. PCR using primer sets around the hypervariable region amplified 0.8, 1.1 and 1.6 kbp fragments for serovars A, B and C, respectively. Alternatively, the 1.6 kbp fragments were amplified with another primer pair encompassing the hypervariable region and was subjected to digestion with *Bgl* II, which resulted in the detection of serovar-specific digestion patterns. These results indicate that PCR and PCR-RFLP using the hypervariable region of *HMTp210* are alternative methods to identify the serovar of *A. paragallinarum*.

KEY WORDS: *Avibacterium paragallinarum*, infectious coryza, multiplex PCR, RFLP.

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Avian infectious coryza is an acute respiratory disease caused by infection with *Avibacterium* (*Haemophilus*) *paragallinarum* (*A. paragallinarum*) with clinical symptoms such as nasal discharge, swelling of the face and epiphora. Infectious coryza has a negative economic impact, since it leads to a decrease in the breeding rate of poultry, failure or retardation of egg laying and a decrease in egg production [1, 2].

Page *et al*. classified *A. paragallinarum* into three serovars, A, B and C [10], whereas Sawata *et al*. classified it into two serovars, 1 and 2 [11]. Subsequently, Kume *et al*. reported that serovar A of Page *et al*. corresponds to serovar 1 of Sawata *et al*., whereas serovar C of Page *et al*. corresponds to serovar 2 of Sawata *et al*. [7, 12]. Moreover, Blackall *et al*. reported that the Kume scheme consists of serovars A, B and C, which correspond to the Page serovars of A, B and C [4]. Currently, serovars A and C of *A. paragallinarum* are considered to be the main causative agents of avian infectious coryza.

To identify the serovar of *A. paragallinarum* using the Page scheme, it is recommended to use hemagglutination-inhibition (HI) tests [3], the procedures of which are complicated and require the use of fresh chicken erythrocytes or chicken erythrocytes fixed with glutaraldehyde. On the other hand, the Kume scheme has not been widely applied, since it is technically demanding to perform and 9 antisera (A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3 and C-4) are required [4].

Chen *et al*. reported a PCR test to detect *A. paragallinarum* [5, 6]. However, the PCR could not identify serovars A, B and C. Soriano *et al*. reported that an enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) could identify each serovar [13], but it would not be easy to analyze the results, since ERIC-PCR showed many patterns even within each serovar. Therefore, we examined more simplified methods using PCR. The *A. paragallinarum* strains used were as follows: 221, 083, W, Georgia and Germany (serovar A); Spross and 0222 (serovar B); and 53–47, Modesto and HK-1 (serovar C).

HMTp210, an outer membrane protein, is a major protective antigen of *A. paragallinarum* [8, 9, 14]. The *HMTp210* gene (GenBank accession number: AR303123.1 for serovar A and AR698445.1 for serovar C) can be divided into three regions based on the DNA sequence homology (Fig. 1). Wu *et al*. reported that regions 1 and 3 are highly conserved between *A. paragallinarum* serovars A and C, respectively [15]. On the other hand, the homology of region 2 is around 50% between serovars A and C. Thus, region 2 seems to be a serovar-specific region in HMTp210, which is the best studied antigen for protection against serovars A and C. For these reasons, we focused on region 2 as the target to identify the serovars of *A. paragallinarum* using PCR and PCR-RFLP methods.

The homology of region 2 was more than 99.8% within each serovar: 99.9% within serovar A, 100% within serovar B and 99.8% within serovar C. On the other hand, the homology of region 2 between the serovar A 221 strain and serovar B 0222 strain or serovar C 53–47 strain was 50%, respectively. In contrast to this, the homology between the serovar B 0222 strain and serovar C 53–47 strain was 98% (data not shown).

From the analysis, PCR primers were designed as shown in Table 1 and Fig. 1. Multiplex PCR using primer sets #1–3 was performed in a total volume of 50 μl containing 1 ×

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Table 1. Primer sets used for amplification of the DNA fragment

Primer set #	Primer	Fragment length	Primer sequence
1	ABC forward A reverse	0.8 kbp	5'-GGCTCACAGCTTTATGCAACGAA-3' 5'-CGCGGGATTGTTGATTTTGTT-3'
2	ABC forward B reverse	1.1 kbp	5'-GGCTCACAGCTTTATGCAACGAA-3' 5'-GGTGAATTTCACCACACCAC-3'
3	ABC forward C reverse	1.6 kbp	5'-GGCTCACAGCTTTATGCAACGAA-3' 5'-TAATTTTCTTATTCCCAGCATCAATACCAT-3'
4	⊿5-1 forward ⊿5-1 reverse	1.6 kbp	5'-GATGGCACAATTACATTTACA-3' 5'-ACCTTGAGTGCTAGATGCTGTAGGTGC-3'

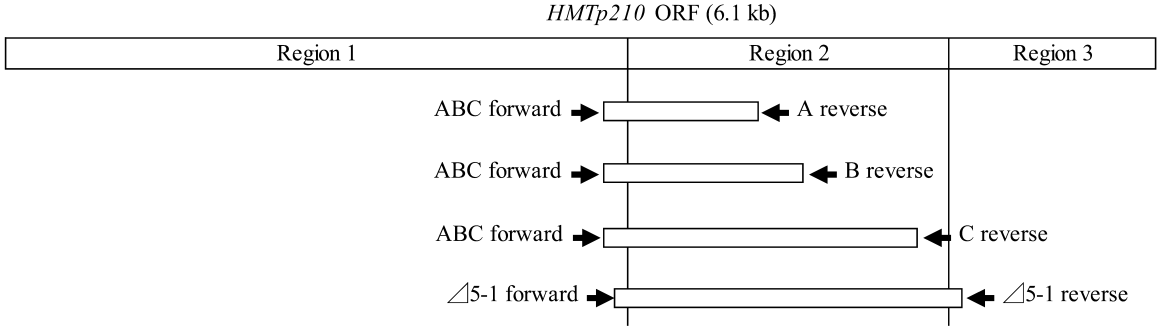

Fig. 1. Location of primers for PCR.

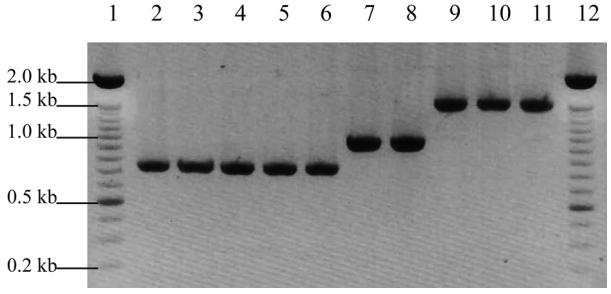

Fig. 2. Agarose gel electrophoresis of multiplex PCR products from 10 strains of *A. paragallinarum.* Marker (lanes 1 and 12); 221, 083, W, Georgia and Germany (serovar A, lanes 2, 3, 4, 5 and 6); Spross and 0222 (serovar B, lanes 7 and 8); and 53–47, Modesto and HK-1 (serovar C, lanes 9, 10 and 11).

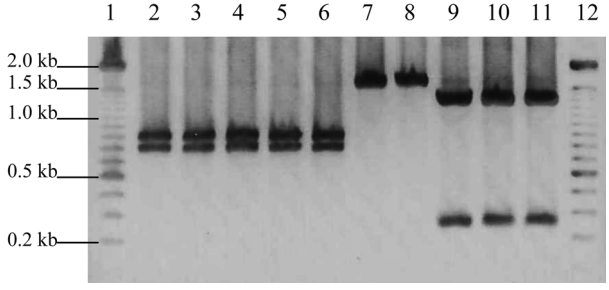

Fig. 3. Restriction fragment length polymorphism (RFLP) patterns of PCR products from 10 strains of *A. paragallinarum* after digestion with *Bgl* II. Marker (lanes 1 and 12); 221, 083, W, Georgia and Germany (serovar A, lanes 2, 3, 4, 5 and 6); Spross and 0222 (serovar B, lanes 7 and 8); and 53–47, Modesto and HK-1 (serovar C, lanes 9, 10 and 11).

PrimeSTAR buffer (Takara Bio Inc., Shiga, Japan), 0.2 mM of each dNTP, 0.2 μM of each serovar-specific primer, 1.25 units of PrimeSTAR DNA Taq polymerase (Takara Bio Inc.) and 100 *n*g of template DNA. The genomic DNA of *A. paragallinarum* was extracted using a Puregene Yeast/Bact. Kit (QIAGEN Corp., Tokyo, Japan) according to the manufacturer's instructions. The amplification steps were as follows: 98°C for 1 min; 30 cycles of 98°C for 10 sec, 56°C for 10 sec and 72°C for 2 min; and a final step at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis.

The results of multiplex PCR are shown in Fig. 2. The sizes of the amplified fragments were 0.8, 1.1 and 1.6 kbp for serovars A, B and C, respectively. Nonspecific amplification was not detected.

In the case of PCR, mutation in the sequences corresponding to the primers may lead to the failure of amplification. To overcome this, PCR-RFLP using another primer pair was developed. First, DNA fragments of approximately 1.6 kbp were amplified using the #4 primer pair in Table 1. The amplification steps were as follows: 98°C for 1 min; 30 cycles of 98°C for 10 sec, 55°C for 10 sec and 72°C for 2 min; and a final step at 72°C for 7 min. Then, the PCR products were digested with the restriction enzyme *Bgl*

II, which resulted in three distinct profiles of restriction fragment length polymorphism (RFLP) for each serovar (Fig. 3). The PCR products were digested into two bands, 768 and 868 bp in the case of serovar A and 1,284 and 339 bp in the case of serovar C. On the other hand, the PCR products from serovar B were not digested. These results indicate that the PCR-RFLP method using the *Δ*5-1 primer pair combined with *Bgl* II digestion can identify *A. paragallinarum* serovars A, B and C, respectively. However, the number of strains examined in this study was small. More strains of *A. paragallinarum* should be analyzed to further evaluate PCR and PCR-RFLP.

Serological diagnosis of the disease has not been performed because the progress of this disease is rapid and antibodies are not likely to be induced in chickens infected with *A. paragallinarum*, particularly with serovar C, even after the disease onset. HI tests are used for identification of *A. paragallinarum*; however, the procedure is complicated, and the sensitivity is insufficient. On the other hand, the newly developed PCR and PCR-RFLP methods provide high accuracy for rapid serotyping of *A. paragallinarum*.

In conclusion, this study shows that multiplex PCR and PCR-RFLP using the hypervariable region of the *HMTp210* gene are alternative methods to identify the serovar of *A. paragallinarum*.

REFERENCES

1. Blackall, P. J. and Soriano, V. E. 2008. Infectious coryza. pp. 789–803. *In*: Disease of Poultry, 12th ed. (Saif, Y. M., Fadly, A. M., Glisson, J. R., McDougald, L. R., Nolan, L. K. and Swayne, D. A. eds.), Blackwell Publishing, Iowa.
2. Blackall, P. J. 1999. Infectious coryza: overview of the disease and new diagnostic options. *Clin. Microbiol. Rev.* **12**: 627–632.
3. Blackall, P. J., Eaves, L. E., and Aus, G. 1990. Serotyping of *Haemophilus paragallinarum* by the Page scheme: comparison of the use of agglutination and hemagglutination-inhibition tests. *Avian Dis.* **34**: 643–645.
4. Blackall, P. J., Eaves, L. E. and Rogers, D. G. 1990. Proposal of a new serovar and altered nomenclature for *Haemophilus paragallinarum* in Kume hemagglutinin scheme. *J. Clin. Microbiol.* **28**: 1185–1187.
5. Chen, X., Miflin, J. K., Zhanq, P. and Blackall, P. J. 1996. Development and application of DNA probes and PCR tests for *Haemophilus paragallinaru*m. *Avian Dis.* **40**: 398–407.
6. Chen, X., Chen, Q., Zhanq, P., Feng, W. and Blackall, P. J. 1998. Evaluation of a PCR test for the detection of *Haemophilus paragallinarum* in China. *Avian Pathol.* **27**: 296–300.
7. Kume, K., Sawata, A. and Nakase, Y. 1980. Immunologic relationship between page's and Sawata's serovar Stains of *Haemophilus paragallinarum*. *Am. J. Vet. Res.* **41**: 757–760.
8. Noro, T., Yaguchi, K., Amimoto, K. and Oishi, E. 2007. Identification and expression of a gene encoding an Epitope that induces hemagglutination inhibition antibody to *Avibacterium paragallinarum* Serovar A. *Avian. Dis.* **51**: 84–89.
9. Noro, T., Oishi, E., Kaneshige, T., Yaguchi, K., Amimoto, K. and Shimizu, M. 2008. Identification and characterization of haemagglutinin epitopes of *Avibacterium paragallinarum* serovar C. *Vet. Microbiol.* **131**: 406–413.
10. Page, L. A. 1962. Haemohilus infections in chickens. 1. Characteristics of 12 Haemohilus isolates recovered from diseased chickens. *Am. J. Vet. Res.* **23**: 85–95.
11. Sawata, A., Kume, K. and Nakase, Y. 1978. Haemophilus infection in chickens 2. Types of *Haemophilus paragallinarum* isolates from chickens with infectious coryza, in relation to *Haemophilus gallinarum* strain No.221. *Jpn. J. Vet. Sci.* **40**: 645–652.
12. Sawata, A., Kume, K. and Nakase, Y. 1980. Biologic and serologic relationships between Page's and Sawata's serovars of *Haemophilus paragallinarum*. *Am. J. Vet. Res.* **41**: 1901–1904.
13. Soriano, V. E., Tellez, G., Hargis, B. M., Newberry, L., Salgado, M. C. and Vazquez, J.C. 2004. Typing of *Haemophilus paragallinarum* strains by using enterobacterial repetitive intergenic consensus-based polymerase chain reaction. *Avian Dis.* **48**: 890–895.
14. Tokunaga, E., Sakaguchi, M., Matsuo, K., Hamada, F. and Tokiyoshi, S. Polypeptide for *Haemophilus paragallinarum* and process for preparing the same. United States Patent No. 6, 919, 080 B2. November 21, 2005.
15. Wu, J. R., Wu, Y. R., Shien, J. H., Hsu, Y. M., Chen, C. F., Shieh, H. K. and Chang, P. C. 2011. Recombinant proteins containing the hypervariable region of the haemagglutinin protect chickens against challenge with *Avibacterium paragallinarum*. *Vaccine* **29**: 660–667.