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Research Note—

Serotyping of *Avibacterium paragallinarum* Isolates from Peru

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SUMMARY. This study appears to represent the first serotyping study of 24 isolates of *Avibacterium paragallinarum* obtained from different regions of Peru during 1998–2008. All isolates were characterized as β-nicotinamide adenine dinucleotide dependent. According to the Page scheme, modified by Blackall, it was found that eight isolates were classified as serogroup A, seven isolates as serogroup B, and five isolates as serogroup C, while four isolates could not be serotyped. Further serotyping, following the same scheme but using rabbit antiserum raised against Argentinean strains of the three serogroups, allowed allocation of these four unclassified isolates to serogroup B. These results suggest that some of the Peruvian B isolates appear to be similar to the previously described variant B isolates from Argentina. Therefore, inactivated vaccines used in Peru should include the three recognized serogroups (A, B, and C), with the addition of at least one of these variant B isolates. Cross-protection trials are needed to compare the protection conferred by vaccines containing traditional B serovar strains to the protection by experimental vaccines containing variant B serovar isolates from Peru.

RESUMEN. *Nota de Investigación*—Serotipificación de aislamientos de *Avibacterium paragallinarum* del Perú.

En este trabajo se presenta el primer estudio de serotipificación de 24 aislamientos de *Avibacterium paragallinarum* obtenidos de diferentes regiones del Perú, durante los años 1998 al 2008. Todos los aislamientos fueron caracterizados como dependientes de la β-nicotinamida adenina dinucleótido. De acuerdo al esquema de Page y modificado por Blackall, se encontró que ocho aislamientos fueron clasificados como serogrupo A, siete aislamientos como serogrupo B y cinco aislamientos como serogrupo C, mientras que cuatro aislamientos no pudieron ser serotipificados. Serotipificaciones posteriores que emplearon el mismo esquema pero con la utilización de antisueros producidos en conejo contra cepas argentinas de los tres serogrupos, permitieron la inclusión en el serogrupo B de cuatro aislamientos no clasificados. Estos resultados sugieren que algunos de los aislamientos peruanos del serogrupo B parecen ser similares a los aislamientos B variantes de Argentina que han sido descritos previamente. Por lo tanto, las vacunas inactivadas utilizadas en el Perú deben incluir los tres serogrupos reconocidos (A, B y C), además de la adición de por lo menos uno de estos aislamientos B variantes. Se requiere de ensayos de protección cruzada para comparar la protección conferida por las vacunas que contienen las cepas tradicionales del serovar B con la protección por vacunas experimentales que contengan los aislamientos variantes del serovar B del Perú.

Key words: *Avibacterium paragallinarum*, chicken, infectious coryza, Peru, serogroup

Abbreviations: CLBA = Columbia agar base plates plus 7% equine hemolyzed blood; NAD = β-nicotinamide adenine dinucleotide; PBS = phosphate buffered saline; PCR = polymerase chain reaction; WB = working buffer

Avibacterium paragallinarum (4) is the etiologic agent of infectious coryza, an upper respiratory tract disease of chickens (*Gallus gallus*) (2,3,36). This disease affects laying hens, breeders, and broilers (23,36). *Avibacterium paragallinarum* is a pleomorphic, Gram-negative bacterium that requires β-nicotinamide adenine dinucleotide (NAD) for *in vitro* growth (36), although in Mexico (22) and South Africa (16), NAD-independent strains have been reported. NAD independence may be acquired through a transmissible plasmid (14). Molecular identification of *Av. paragallinarum* by means of polymerase chain reaction (PCR) HP-1 and PCR HP-2, using primers F1/R1 and N1/R1, respectively, has been described (17). Likewise, a molecular technique, based on the restriction fragment length polymorphism of the 16S ribosomal genes amplified by a PCR and referred to as amplified 16S ribosomal DNA restriction analysis, was designed to identify 19 *Av. paragallinarum* strains isolated from Peru (27).

In laying hens, egg production can be reduced up to 40%, mainly in multiple-age farms (9), when the birds reach the egg-production peak. In broilers, the economic impact of the infection is due to weight loss, an increased number of culls, and a tendency to acquire chronic, complicated respiratory diseases (20,33,37).

The most widely used serologic classification scheme for *Av. paragallinarum* is the Page agglutination scheme (28). Using this scheme, a total of three different serovars, termed A, B, and C, are recognized. By means of inhibition–hemagglutination tests, Kume *et al.* (26) identified seven serovars (HA-1 to HA-7), distributed into three serogroups (termed I, II, and III), in a scheme now known as the Kume scheme. Blackall *et al.* (7) recognized the connections between the Kume and Page schemes and re-organized the Kume scheme to consist of three serogroups and nine serovars. The revised Kume scheme consisted of three serogroups that matched the three serovars of the Page scheme, with serogroup A consisting of 4

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Table 1. Field information and serotyping results for the 24 *Avibacterium paragallinarum* isolates used in this study.

Strain number	Isolation date	Province (City)	Chicken type	Serotype
01	Apr 1998	La Libertad (Trujillo)	Laying hens	B[A]
02	Jan 1999	Ica (Chincha)	Laying hens	B[A]
03	Dec 1999	La Libertad (Trujillo)	Laying hens	B[A]
04	Dec 1999	Arequipa (Arequipa)	Broiler	B[A]
05	Feb 2001	Lima (Huacho)	Breeder	A
06	Mar 2001	Lima (Cañete)	Laying hens	C
07	Apr 2001	Lima (Ate)	Laying hens	C
08	May 2001	Loreto (Iquitos)	Broiler	C
09	Aug 2001	Lima (Ate)	Laying hens	A
10	Aug 2001	Lima (Ate)	Laying hens	B
11	Nov 2001	Loreto (Iquitos)	Broiler	C
12	Feb 2002	La Libertad (Pacasmayo)	Breeder	A
13	Mar 2002	Lima (Chancay)	Broiler	A
14	May 2002	Lima (Puente Piedra)	Laying hens	A
15	Nov 2002	La Libertad (Trujillo)	Laying hens	B
16	Nov 2002	La Libertad (Trujillo)	Laying hens	B
17	Dec 2002	Lima (Cañete)	Laying hens	A
18	Apr 2004	Lima (Lima)	Laying hens	B
19	Dec 2005	Loreto (Iquitos)	Broiler	B
20	Jan 2006	Piura (Piura)	Broiler	C
21	Mar 2006	Lima (Pachacamac)	Broiler	B
22	Jul 2006	Lima (Pachacamac)	Laying hens	A
23	Oct 2007	Lima (Puente Piedra)	Laying hens	B
24	Apr 2008	Lima (Puente Piedra)	Laying hens	A

[A]These isolates were serotyped using Argentinean antiserum of the three Page's serogroups A, B, and C, due to the absence of reaction with the international reference antiserum. Strains 1–4, 18, 19, and 21–24 were provided by Innova Andina S.A. Strains 5–17 and 20 were provided by Bioservice S.R.L.

serovars (A1, A2, A3, and A4), serogroup B having only one serovar (B1), and serogroup C having 4 serovars (C1, C2, C3, and C4) (6). This combined scheme is very useful and flexible because it allows the incorporation of new serovars (6). Serotyping in each geographic area or country is very important for the formulation of inactivated vaccines. In general, using inactivated vaccines, there is cross-protection among strains from the same Page serovars–Kume serogroups (7,35), with the exception of some strains, i.e., those classified as variants of Page serovar B (24,38) and those as Kume serovar C-3 (12,15). In Argentina (37), Brazil (8), Egypt (1), Germany (21), Indonesia (30), Mexico (34), South Africa (16), Spain (29), and the United States (28), the three recognized Page serovars are present. In Australia (5,39) and Japan (25,41), only Page serovars A and C have been reported; in China, only serovars A (18) and B (44); and in Malaysia (42), only serovar A has been reported.

In Peru, infectious coryza is prevalent among the twelve provinces with significant poultry production: Ancash, Arequipa, Ica, La Libertad, Lambayeque, Loreto, Lima, Moquegua, Piura, San Martin, Tacna, and Ucayali. Most production is concentrated in the province of Lima. Despite the fact that infectious coryza is known to exist in Peru in laying hens, breeders, and broilers, the present study appears to represent the first serotyping study of *Av. paragallinarum* isolates from this country.

MATERIALS AND METHODS

Bacteria. A total of 24 isolates of *Av. paragallinarum* from diseased laying hens, broilers, and breeders was examined (Table 1). All isolates were obtained during 1998–2008 from typical clinical cases of infectious coryza; they were isolated from infraorbital sinus and nasal turbinate bone samples. Fourteen lyophilized isolates (numbers 5–17 and 20) were kindly provided by the Bioservice S.R.L., Lima, Peru; and 10 isolates, as fresh culture in modified GC agar slope (DIFCO 228950; Difco, Sparks, MD) were kindly provided by Innova Andina S.A., Lima, Peru. Reference strains of *Av. paragallinarum* (31) 0083 (serovar A), 0222 (serovar B), and Modesto (serovar C) were kindly provided by the Animal Research Institute, Yeerongpilly, Australia. *Avibacterium paragallinarum* strains H23 (serovar A), H8 (variant serovar B) (11), and H32 (serovar C) were obtained from infectious coryza outbreaks in Argentina (23,33,38). Other bacterial strains, such as *Av. paragallinarum* serovar A (ATCC 29545), *Avibacterium gallinarum* (99/003), *Ornithobacterium rhinotracheale* (ATCC 51463), *Pasteurella multocida* (99/213), *Salmonella enteritidis* (86/360), *Escherichia coli* (ATCC 15222), and *E. coli* (EC-02), were used as controls for biochemical and molecular studies. Reference and regional strains are held at the laboratories of the Instituto Nacional de Tecnología Agropecuaria (INTA), Balcarce, Argentina; Innova Andina S.A.; and Bioservice S.R.L., Lima, Péru.

Culture media and characterization tests. Agar GC, supplemented with 25 μg/ml of NADH (N7004; Sigma, St. Louis, MO), 5% inactivated chicken serum, and 1% Iso VitaleX™ enrichment (211876; BBL, Sparks, MD) was used to reconstitute the lyophilized strains and for the transportation of fresh cultures. Columbia agar base (CM331; Oxoid, Basingstoke, England), plus 7% equine hemolyzed blood (CLBA) (36,37) and TM/SN broth supplemented with 25 μg/ml of NADH, 1% inactivated chicken serum, and 5% oleic-albumin complex (7), were used for the preparation of antigens. All strains were biochemically characterized according to Terzolo *et al.* (37).

Molecular identification. Genomic DNA from *Av. paragallinarum* was extracted by the phenol–chloroform method (27,32,43). Species-specific PCR was performed as previously described by Mendoza-Espinoza *et al.* (27). All isolates were tested using N1/R1 primers described by Chen *et al.* (17).

Antisera. Rabbit antisera were raised according to Thornton and Blackall (39) against reference strains of *Av. paragallinarum* 0083, 0222, and Modesto (31) and Argentinean strains H23, H8, and H32 (33,38). Briefly, each antigen was grown overnight onto TM/SN, centrifuged at 4800 $\times$ *g* for 15 min at 4 C, and re-suspended in phosphate buffered saline (PBS) pH 7.2 with 100 μg/ml thimerosal, and adjusted to a McFarland tube 5. Two rabbits per strain were injected at 3-day

intervals; subcutaneous injections of 1 ml of antigen, homogenized in incomplete Freund's adjuvant (F5506; Sigma), was followed by six intravenous injections of the antigen (0.5 ml, 1 ml, 2 ml, 3 ml, 4 ml, and 4 ml). One week after the final injection, rabbits were anesthetized with 16 mg/kg sodium thiopental (Abbott Laboratories, Abbott Park, Chicago, IL) and exsanguinated by cardiac puncture. The serum of each strain was pooled, lyophilized, and kept at −20 C.

Preparation of antigens for serotyping tests. *Avibacterium paragallinarum* strains were microaerophilically grown onto CLBA plates (36), which were used to seed 600 ml of supplemented TM/SN broth. After incubation, the broth was centrifuged twice at 4800 × *g* for 15 min at 4 C. The pellet was suspended in 1 ml PBS plus thimerosal (100 μg/ml) and centrifuged again at 5000 × *g* for 2 min at 4 C. The pellet was then re-suspended in 4 ml of PBS plus thimerosal and kept at 4 C until usage.

Preparation of chicken erythrocytes. Glutaraldehyde-fixed erythrocyte suspension (30%) in distilled water plus 100 μg/ml of thimerosal was prepared according to Eaves *et al.* (21) and kept in darkness at 4 C until usage. When used, this solution was diluted 1:100 in a working buffer (WB) composed of PBS (pH 7.2) containing 0.1% of bovine serum albumin and 0.001% of gelatin (10).

Hemagglutination tests. All isolates were serotyped according the Page scheme (28), following the hemagglutination-inhibition test of Eaves *et al.* (21). Briefly, serial 2-fold dilutions of 50 μl in WB of each antigen were made. To each dilution, 50 μl of erythrocytes were added and incubated 1 hr at room temperature.

Hemagglutination inhibition tests. Serial, 2-fold dilutions in 50 μl WB were prepared for each antiserum. To each dilution, 50 μl of four hemagglutination units (21) of the corresponding antigen were added. After 30 min of incubation at room temperature, 50 μl of erythrocytes were added. After another 30 min of incubation at room temperature, the serotype of each antigen was assessed as the highest titer of hemagglutination inhibition. All isolates that could not be serotyped by using the sera raised against reference strains 0083, 0222, and Modesto were re-tested using the sera raised against Argentinean strains H23, H8, and H32.

RESULTS AND DISCUSSION

This study appears to represent the first serotyping study of isolates of *Av. paragallinarum* from Peru. All isolates were classified as NAD-dependant *Av. paragallinarum*, and all biochemical and molecular tests were found to be typical of this species (17,37). The origin of each isolate, type of production, and isolation dates are described in Table 1.

According to the Page scheme (28), as modified by Blackall (6), using the rabbit antiserum raised against reference strains 0083 (serovar A), 0222 (serovar B), and Modesto (serovar C), it was established that eight isolates were classified as serovar A, seven isolates as serovar B, and five isolates were classified as serovar C; four isolates (isolates 1, 2, 3, and 4) could not be serotyped (Table 1). Nevertheless, further serotyping following the same scheme, although using rabbit antiserum raised against Argentinean strains H23 (serovar A), H8 (serovar B), and H32 (serovar C), allowed allocation of these unclassified isolates 1, 2, 3, and 4 as belonging to serovar B. It was uncommon to find serovar B strains as a cause of infectious coryza outbreaks all over the world until they were described in Argentina (37) in 1993 as a prevalent serovar. These strains were very pathogenic, able to invade other organs beyond the upper respiratory tract in laying hens, breeders, and broilers, and were thus able to cause serious septicemia and arthritis (33). Comparing the aforementioned four Argentinean B isolates by multilocus enzyme electrophoresis with 118 strains from 5 continents around the world (11), it was found that these strains were allocated in a separate cluster and were different from the other strains, including the other serovar B isolates. The strain H8, which was used in this work to produce antiserum, was one of these four strains. At present, variant B strains have been described in Argentina, Ecuador, the United States, and Zimbabwe (24,33). In challenge trials aimed at evaluating the protection rendered by different commercial and experimental vaccines, it has been demonstrated that there was limited cross-protection between standard and variant serogroup B strains (24,38). Therefore, in order to afford protection, it is of high importance to detect the presence of variant B strains in a determined geographic area and, accordingly, include them in regional vaccines.

Thirteen out of 24 isolates have been found from Lima Province, where the majority of Peruvian poultry production is located and where all three serovars were recognized. The other 11 isolates were obtained from five other provinces (Table 1). Variant B serovars were found in the provinces of Arequipa, Ica, and La Libertad. Further work should be carried out in order to study additional infectious coryza outbreaks from all of the Peruvian provinces with a poultry industry. In this way, the real geographic distribution of all serovars might be obtained.

The control of infectious coryza mainly relies on vaccination using inactivated vaccines. In areas where infectious coryza is prevalent, it is advisable to combine vaccination with the administration of disinfectants in order to reduce environmental exposure (13,23). It has been demonstrated that such a procedure effectively reduces the clinical signs of disease (13,23). The sole application of a treatment with antibiotics is not advisable because most drugs have a bacteriostatic rather than bactericidal action and, thus, the disease persists in carrier birds. These carrier animals maintain the infection in the farm for a long time and are able to infect young, susceptible animals when they are introduced into the farms (9). Re-infection in this way is very frequent in multiple-age farms, which is a common poultry rearing method used in Peru as well as in other South-American countries (9). Furthermore, at present, the usage of some antibiotics is forbidden in poultry production due to the increasing bacterial resistance. Moreover, eggs and chicken meat for human consumption should be free from chemical drugs (19,40).

Up to now, all variant B strains have been serotyped using international reference B antisera (24,37). In this study, the four variant B strains from Peru could not be serotyped using the standard B antisera, while they could be allocated as serovar B by using the antisera raised against an Argentinean variant B isolate. These results suggest that these Peruvian isolates might be less related to the reference B strain than to the Argentinean variant B strain. Consequently, inactivated vaccines to be used in Peru should include the three recognized serogroups (A, B, and C) together with the Peruvian variant B strains. At the same time, more studies should be carried out, particularly cross-protection trials, to determine if these serologic differences really represent differences that are significant in terms of vaccine protection.

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