***Haemophilus* Infections in Chickens**

2. Types of *Haemophilus paragallinarum* Isolates from Chickens with Infectious Coryza, in Relation to *Haemophilus gallinarum* Strain No. 221

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Abstract. Eighty-eight *Haemophilus* isolates derived from chickens with infectious coryza were classified. Then they were compared biologically and serologically with *Haemophilus gallinarum* type I and III of strain No. 221 described by Kato et al., and with *H. paragallinarum* strain H-18 reported previously by the authors. Of the isolates, 86 were capsulated and biologically identified as *H. paragallinarum*. Of them, 62 strains were hemagglutinable and serologically identical with *H. gallinarum* type I of strain No. 221 (*H. paragallinarum* serotype 1) and 24 strains unhemagglutinable and serologically identical with *H. paragallinarum* strain H-18 (*H. paragallinarum* serotype 2). All the capsulated strains corresponded to either serotype 1 or 2, which showed a similar degree of pathogenicity for chickens, irrespective of serotype. The remaining 2 isolates were noncapsulated and nonpathogenic, and identical with Kato's type III strain, which was a variant derived from capsulated *H. paragallinarum* serotype 1.

Since 1962, attempts have been made to classify *Haemophilus* isolates derived from chickens with infectious coryza (IC) [2, 7, 8, 12]. Recently, the authors [11, 15] reported *Haemophilus paragallinarum* (Hpg) strains antigenically distinct from *Haemophilus gallinarum* (Hg) strain No. 221 (Hg 221) [7], which had been used for the preparation of infectious coryza vaccine (ICV) [5].

This paper deals with the typing of Hpg isolates from chickens with coryza on several Japanese poultry farms and with the relationships between the types of Hpg and Kato's types I and III of Hg [7, 8].

Materials and Methods

Source of strains: In 1976-77 the authors isolated 88 *Haemophilus* strains from the paranasal sinus or nasal cavity of 51 chickens affected with IC on 5

poultry farms and submitted to our laboratory for diagnosis. All these strains were used (Table 1).

Reference strains: Hg 221 (Kato's type I strain; Hg 221-I) [7, 8] supplied kindly by Dr. Kato, Hpg strain H-18 (Hpg H-18) isolated by the authors [11, 15], and strain 9899 (Kato's type III strain; Hg 221-III) obtained from a chicken infected experimentally with Hg 221-I [8] were used as reference strains.

Isolation and identification: By the techniques described in the previous paper [11] *Haemophilus* organisms were isolated. Cultures were obtained from individual colonies. The isolates were identified biologically by the procedures reported in the previous paper [11].

Hemagglutinating activity: Hemagglutinating (HA) activity was examined by the method of Kato et al. [8].

Chickens: Hen's eggs from grandparent stock H and N (Shizuoka-Ken, Japan), known to be free from *Haemophilus* organisms and certain bacterial and viral infections, were incubated in the laboratory. The hatched chicks were used for pathogenicity and immunologic tests.

鶏のヘモフィルス感染症に関する研究 II. 伝染性コリザ発症鶏から分離されたヘモフィルス・パラガリナラムの菌型ならびにヘモフィルス・ガリナラム 221 株との関係: 沢田 章・久米勝巳・中瀬安清 (北里研究所)

Table 1. Isolation of *Haemophilus paragallinarum* strains from outbreaks of infectious coryza on five poultry farms

Farm	A		B		C		D		Total
Area	Iwate		Aomori		Gumma		Iwate	Aomori	
Size of flock	12,000		10,000		2,700 3,000		24,000	10,000	61,700
Date	Dec. 76	Sept. 76	Nov. 76	May 76	Feb. 77		Oct. 76	Sept. 76	
Age in days	245		192*	265*	330	168	181	418	
Identification of chicken group	A		B-1	B-2	C-1	C-2	D	E	
History of vaccination** (days)									
First	70		50	50	52	50	35	70	
Second	150		140	140	92	100	105	140	
HI titer against 221 antigen (GM)	17.5		12.6	10.8	≤2.5	26.8	15.8	≤2.5	
Days after onset of clinical coryza signs	35		7	3	4	3	22	4	
Morbidity† (%)	65-85		80-90	4-7	20-60	5-7	5-9	7-15	
Culture positive rate	5/5		9/10	10/10	4/6	5/5	5/5	10/10	
<i>Haemophilus</i> strains obtained‡	9		16	20	3	10	10	20	88

Remarks.

*: The same flock.

**: Vaccination with a commercial infectious coryza vaccine prepared from *Haemophilus gallinarum* strain No. 221.

†: Infected chickens with nasal discharge and/or facial swelling.

‡: Two *Haemophilus*-like colonies, except for C-1 group (1 colony), were fished from each bird.Catalase-positive *Haemophilus* was excluded from the result.

HI: Hemagglutination-inhibition test. GM: Geometric mean.

Pathogenicity test: Seven-week-old chickens were administered with 0.2 ml of culture suspension (at least 10^8 colony-forming units/0.2 ml/chicken) by the nasal route. Clinical findings, nasal discharge, and facial swelling were recorded daily in each chicken for 1 week after inoculation.

Preparation of antigen and antiserum: Agglutininogen was prepared by the method as reported by the authors [11]. Hyperimmune serum was obtained from a rabbit injected with diluted antigen (1 billion/ml) 6 times at 1-week intervals.

Agglutination test: The rapid plate method (RPA) [2, 10, 11, 17] was performed with antiserum diluted 1:25 or 1:800 in phosphate buffered saline.

Hemagglutination-inhibition (HI) test: By using HA-antigen prepared with Hg 221-I, HI antibody was determined by the same method as described by Kato [6].

Antibody formation test in chickens: Thimerosal-inactivated vaccine (5 billion/ml) with $Al(OH)_3$

(900 µg/ml) as adjuvant was prepared by the method mentioned in the previous paper [11]. Chickens were injected with a single dose (1 ml) of it at 4 weeks of age or with three doses at 4, 7 and 10 weeks of age, respectively. Antisera obtained 3 weeks after the final injection were tested by the RPA method.

Results

Isolation of *Haemophilus* organisms from chickens on 5 farms in the outbreak of IC: Table 1 shows the outbreaks of IC in chickens routinely vaccinated with commercial ICV prepared from killed Hg 221 on 5 farms. On farm B, the same chicken flock was re-infected with IC about 70 days after recovery from the first infection with coryza. On farm C, 2 flocks at different age

Table 2. Biologic properties and pathogenicity of *Haemophilus* strains

Chicken group	A	B-1	B-2	C-1	C-2	D	E	Total
No. of <i>Haemophilus</i> strains tested	9	16	20	3	10	10	20	88
Strain No.	7682-7690	6501-6516	7119-7138	7712-7714	7411-7420	7159-7168	7301-7320	88
Capsule :								
No. of positive strains	9	16	20	3	10	8 (7161-68*)	20	86
No. of negative strains	0	0	0	0	0	2 (7159-60)	0	2
No. of strains required for growth :								
X factor (Hemin=50 µg)	0	0	0	0	0	0	0	0
V factor (DPNH=5 µg)	9	16	20	3	10	10	20	88
Chicken serum (1%)	9	16	20	3	10	8 (7161-68)	20	86
Hemagglutinating activity :								
No. of positive strains	4 (2**)	0	20 (10)	0	10 (5)	8 (5†)	20 (10)	62 (32)
strain No.	(7682-85)					(7161-68)		
No. of negative strains	5 (3**)	16 (9)	0	3 (3)	0	2 (2†)	0	26 (17)
strain No.	(7686-90)					(7159-60)		
No. of strains with pathogenicity for 7-week-old chickens	9	16	20	3	10	8 (7161-68)	20	86

Remarks.

* : Strain No. showing properties indicated.

** : Number of chickens isolated. *Haemophilus* strains, isolated from each bird, showed similar hemagglutinating activity.† : Both hemagglutinable and unhemagglutinable *Haemophilus* strains were isolated from 2 birds.

DPNH : Diphosphopyridine nucleotide.

were simultaneously infected with different types of *Haemophilus*. The chickens of all the groups, except groups C-I and E, had HI antibody against Hg 221-I, the titer of which ranged from 1:10.8 to 1:26.8 as geometric mean. Isolation of organisms was tried in 51 chickens of 7 groups 3 to 35 days after the onset of clinical signs of coryza. A total of 88 *Haemophilus* isolates were obtained from 48 chickens.

Biologic properties and pathogenicity of *Haemophilus* isolates: Table 2 shows the biologic properties and pathogenicity of the 88 isolates examined. Of these isolates, 86 strains were capsulated and formed iridescent colonies on S agar medium by 12 hours of incubation. Their morphologic findings were similar to those of either Hg 221-I or Hpg H-18. The remaining 2 noncapsulated strains, Nos. 7159 and 7160,

were derived from 2 chickens on farm D and produced noniridescent colonies, presenting similar morphologic findings to those of strain 9899 (Hg 221-III). None of the isolates required both V and X factors for growth. All the capsulated strains, as well as Hg 221-I and Hpg H-18, required V factor and chicken serum, but not X factor, for growth. All the noncapsulated strains, as well as strain 9899, required only V factor for growth.

All the isolates belonged to the catalase-negative group, reduced nitrates, but did not produce indol. Strains 7159 and 7160, as well as strain 9899, fermented both mannitol and sorbitol, but the capsulated strains, as well as Hg 221-I and Hpg H-18, failed to do so.

The 88 isolates were divided into 2 groups by HA activity. Of the 86 capsu-

Table 3. Cross-agglutination tests between isolates and reference strains

Chicken group	Strain No.	Anti-sera and dilutions used											
		Anti-Hg 221-I		Anti-Hpg 7119		Anti-Hpg H-18		Anti-Hpg 6508		Anti-9899		Anti-7160	
		×25	×800	×25	×800	×25	×800	×25	×800	×25	×800	×25	×800
A	7682	++*	++	++	++	++	—**	+†	—	+	—	+	—
	7685	++	++	++	++	+	—	+	—	+	—	+	—
	7686	+	—	++	—	++	++	++	++	+	—	+	—
	7690	+	—	+	—	++	++	++	++	+	—	++	—
B-1	6501	++	—	+	—	++	++	++	++	+	—	+	—
	6516	+	—	+	—	++	++	++	++	+	—	++	—
B-2	7119	++	++	++	++	+	—	+	—	+	—	+	—
	7138	++	++	++	++	++	—	+	—	+	—	+	—
C-1	7712	+	—	+	—	++	++	++	++	+	—	+	—
	7714	+	—	+	—	++	++	++	++	+	—	+	—
C-2	7411	++	++	++	++	+	—	+	—	++	—	+	—
	7420	++	++	++	++	+	—	+	—	+	—	+	—
D	7159	+	—	+	—	++	—	+	—	++	++	++	++
	7160	+	—	+	—	+	—	+	—	++	++	++	++
	7161	++	++	++	++	+	—	+	—	+	—	+	—
	7168	++	++	++	++	+	—	+	—	+	—	+	—
E	7301	++	++	++	++	+	—	+	—	++	—	+	—
	7320	++	++	++	++	+	—	+	—	+	—	+	—
Reference strain	Hpg H-18	+	—	+	—	++	++	++	++	+	—	+	—
	Hg 221-I	++	++	++	++	+	—	+	—	+	—	++	—
	9899	+	—	+	—	+	—	+	—	++	++	++	++

Remarks.

*: Agglutinated within one minute.

**: Agglutinated within one to two minutes.

†: Non-agglutinated.

Hpg: *Haemophilus paragallinarum*. Hg: *Haemophilus gallinarum*.

lated strains, 62, as well as Hg 221-I, agglutinated red cells of chicken, sheep, cattle, horse, and guinea pig. The remaining 24 capsulated and the 2 noncapsulated strains, as well as Hpg H-18, failed to have such activity. Both hemagglutinable and unhemagglutinable strains, including the capsulated and noncapsulated ones, were obtained simultaneously from 2 of 5 chickens on farm D.

All the capsulated Hpg isolates, as well as Hg 221-I and Hpg H-18, were highly pathogenic. No significant differences were observed in pathogenicity among these strains. All the noncapsulated strains, 7159, 7160 and 9899, however, were found to be avirulent.

Cross-agglutination tests between isolates and reference strains: Cross-agglutination tests were performed between isolates and reference strains. Table 3 shows the results of the agglutination test with representative isolates. In 1:800 serum dilution, the isolates, as well as the reference strains, Hpg H-18, Hg 221-I and strain 9899, were divided into 3 groups. Cross-agglutination was observed among these strains in 1:25 serum dilution.

Serologic properties of *Haemophilus* isolates: The results of the RPA tests among antigens prepared with the individual isolates and specific anti-Hg 221-I, anti-Hpg 7119, anti-Hpg H-18, anti-Hpg 6508, anti-9899 and anti-7160 sera are summarized in

Table 4. Serologic properties of *Haemophilus* strains

Chicken group	A	B-1	B-2	C-1	C-2	D	E	Total	Reference strain	
No. of strains tested	9	16	20	3	10	10	20	88	Hpg H-18	Hg 9899
Strain No.	7682-7690	6501-6516	7119-7138	7712-7714	7411-7420	7159-7168	7301-7320			
RPA, No. of positive strains against specific (1 : 800)										
Hg 221-I antiserum	4 (7682-85*)	0	20	0	10	8 (7161-68)	20	62	-**	+†
Hpg 7119 antiserum	4 (7682-85)	0	20	0	10	8 (7161-68)	20	62	-	+
Hpg H-18 antiserum	5 (7686-90)	16	0	3	0	0	0	24	+	-
Hpg 6508 antiserum	5 (7686-90)	16	0	3	0	0	0	24	+	-
9899 antiserum	0	0	0	0	0	2 (7159-60)	0	2	-	+
7160 antiserum	0	0	0	0	0	2 (7159-60)	0	2	-	+
Antibody producing ability (≥ 5) in chickens. No. strains produced (1 dose) :										
HI antibody	4 (7682-85)	0	20	0	10	8 (7161-68)	20	62	-	+
anti-Hg 221-I†	4 (7682-85)	0	20	0	10	8 (7161-68)	20	62	-	+
anti-Hpg 7119‡	4 (7682-85)	0	20	0	10	8 (7161-68)	20	62	-	+
anti-Hpg H-18‡	5 (7686-90)	16	0	3	0	0	0	24	+	-
anti-Hpg 6508‡	5 (7686-90)	16	0	3	0	0	0	24	+	-
anti-9899‡	0	0	0	0	0	2 (7159-60)	0	2	-	+
anti-7160‡	0	0	0	0	0	2 (7159-60)	0	2	-	+

Remarks.

* : Strain No. showing properties indicated.

** : Non-agglutinated.

† : Agglutinated within two minutes.

‡ : Agglutinin.

RPA : Rapid plate agglutination test. Hpg : *Haemophilus paragallinarum*. Hg : *Haemophilus gallinarum*. HI : Hemagglutination-inhibition test.

Table 4. The 62 hemagglutinable Hpg isolates, as well as Hg 221-I, belonged to one serologic type (Hpg serotype 1, Hpg type 1), while the 24 unhemagglutinable Hpg isolates, as well as Hpg H-18, belonged to another serologic type (Hpg serotype 2, Hpg type 2). The remaining strains, 7159, 7160 and 9899, indicated a similar serologic reaction to one another and were distinguished from both Hpg types 1 and 2.

HI antibody and anti-Hg 221-I and anti-Hpg 7119 agglutinins were detected in chickens which had been injected with some isolates belonging to Hpg type 1. On the other hand, anti-Hpg H-18 and anti-Hpg 6508 agglutinins were produced in chickens injected with some isolates belonging to Hpg type 2. Chickens injected with strain 7159, 7160 or 9899 at a 3-dose level produced only anti-9899 and anti-7160 agglutinins. When injected with 3 doses of a strain belonging to either Hpg type 1 or 2, chickens produced not only antibody specific for one type but also antibody common to both types.

Discussion

Kato et al. [7, 8] indicated their type I strains of Hg characterized by HA activity as main causes of IC in Japan. Hinz [1] isolated Hpg strains from chickens infected with IC, and found Hpg to be a causative agent of IC in chickens. The authors [11, 15] also reported that *Haemophilus* strains derived from chickens recently infected with IC were different from Hg and could be identified as Hpg. There was a difference in hemagglutinability between Kato's type I strains of Hg and the authors' Hpg strains [11, 15]. These Hpg strains belonged to a single serologic type, which was antigenically different from Hg 221-I.

In the present investigation, an attempt was made to classify the virulent capsulated

strains of *Haemophilus* so far isolated from birds with coryza, and to compare these with Hpg H-18 [11], unhemagglutinable Hg 221-III (strain 9899), and hemagglutinable Hg 221-I. All the capsulated *Haemophilus* isolates were identified biologically as Hpg because of the independence from X factor. The capsulated Hpg strains were divided by the HA test into 2 groups, hemagglutinable and unhemagglutinable, and by the RPA test into 2 serologic types designated Hpg serotypes 1 and 2 (Hpg types 1 and 2) temporarily.

Hinz [2] reported the antigenic types of Hpg to be serotypes A and B, which were identical as Page's types A and B of Hg, respectively [12, 13]. Kato and Tsubahara [6] divided the Hg strains into 3 serologic groups, types I, II and III, and confirmed type I of Hg, including Hg 221-I, to correspond to Page's Type A. The authors proposed that Hg 221-I should be named *paragallinarum*, though it had not been confirmed to meet the initial requirement of X factor of Hg type I strains by Kato and Tsubahara [7]. They also indicated that the virulent Hpg strains could be classified at least 2 serotypes, though it was not certain whether the authors' Hpg type 2 was identical with Hinz's type B or Page's type B or C.

It was found that Hpg serotypes corresponded to these groups by HA property. Namely, all the Hpg type 1 strains were hemagglutinable and produced HI antibody against Hg 221 HA-antigen, while all the Hpg type 2 strains were unhemagglutinable and produced no HI antibody in chickens. The HA test was considered to be a simple and useful method for classification of capsulated Hpg strains into two serotypes, 1 and 2.

On S agar medium [11], noniridescent *Haemophilus* colonies [3] were isolated from

2 chickens on farm D. The morphologic, biologic and serologic properties, as well as pathogenicity, of these strains were similar to those of Hg 221-III, which had been obtained by the authors [16] from chickens experimentally infected with hemagglutinable Hg 221-I, by the method of Kato et al. [8]. Hinz [3] reported 2 phenotypic forms, M variant (mucoid) and R variant (rough), of a strain of his Hpg type B. He found that R to M or M to R variations occurred in specific-pathogen-free chickens following intranasal inoculation of organisms of R or M cultures. In contrast, Kato et al. [8] considered their groups, classified by HA activity, to be serotypes. Not only in serologic properties but also in morphologic and biologic properties, there were differences between Hg 221-III and Hpg type 1 or 2 strains. The authors propose that Kato's type III strains should be referred to as a variant from his type I.

Antigens prepared with 2 noncapsulated strains, as well as strain 9899, reacted only to anti-9899 and anti-7160 antisera. Chickens injected with these strains failed to produce any type-specific antibody. Anti-9899 and anti-7160 agglutinins were considered to be against such common antigen as described by Page [12] and Hinz [2]. These results also supported a proposal that noncapsulated *Haemophilus* strains, isolated from infected chickens, should be referred to as a variant. Further experiments are necessary to clarify the antigenic relationship between both serotypes of Hpg strains and their phenotypic forms.

The variant was different from *Haemophilus avium* [4], a catalase-positive avirulent *Haemophilus* [1, 9, 11, 12, 14], in colonial morphology.

Evidence is apparent that not only the serotypes of Hpg strains but also their variants were isolated from chickens in-

fectured with IC in the field. Phenotypic forms derived from the Hpg type 1 strain corresponded well to the groups classified by HA property. Variants of Hpg type 2 strain may be distinguished by their colony type. Although Kato et al. [7, 8] proposed their serologic types, the results of the present investigation suggest that their antigenic type of Hg may involve both serotype and its variant forms.

The authors have never isolated the two serotypes of Hpg simultaneously from a bird, but found that both serologic types of Hpg were mixed in an epizootic. Because of the difference in immunogenicity between Hpg types 1 and 2, as previously reported by the authors between Hpg H-18 and Hg 221-I [10, 11, 15], a need for bivalent vaccine, composed of both serotypes of Hpg, is suggested to improve the control of IC by vaccination in the field.

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