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Haemophilus Infections in Chickens. 3. Immunogenicity of Serotypes 1 and 2 Strains of *Haemophilus paragallinarum*

Katsumi KUME, Akira SAWATA and Yasukiyo NAKASE

The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108

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Abstract. Immunogenicity of *Haemophilus paragallinarum* strains belonging to Sawata's serotypes 1 and 2 was investigated. Eleven isolates tested could be divided into two immunotypes which correlated closely with serotype-specificity, one corresponding to strain 221 of serotype 1 and the other to H-18 of 2. No cross-protection was observed between these immunotypes, even though in the hyper-immunized chickens. The bacterins prepared from serotype 2 strains never produced hemagglutination-inhibition antibody, but gave protection to the chickens against challenge-exposure with homologous type strains. Significantly individual variation in the agglutinin production was observed among the chickens inoculated with single dose, which had enough protection. There was a tendency that its protection rate was increased depending on the dosage of primary inoculation.

Several workers have so far reported the presence of cross-protection among Page's serotypes [12, 13] of *Haemophilus paragallinarum* in varing extent [1, 2, 10, 14, 15]. However, since Matsumoto and Yamamoto [10], the presence of type-specific immunity among the serologic types has been reported [1, 2, 14, 15]. We [16] reported the presences of two different serotypes 1 and 2 among *H. paragallinarum* isolates, and of their type-specific antigens [17]. Subsequently, we [8, 9] described the serotype-specific immunity without cross-protection between the serotype 1 strain 221 [6] and the serotype 2 strain H-18 [7].

This paper deals with further study of the immunogenicity of the freshly isolated *H. paragallinarum* strains belonging to the serotypes 1 and 2.

Materials and Methods

Strains and cultures: *H. paragallinarum* Sawata's serotype 1 strains 7682, 7719, 7411, 7161 and 7301, and serotype 2 H-18, 1101, 7710, FO-8, K-6 and FO-26 were isolated in our laboratory [7, 16], and used for the experiment before the fifth subculture postisolation. *H. paragallinarum* strain 221 [6], supplied kindly by Dr. Kato, National Institute Animal Health, Niigata-ken, Japan, was also used as a reference strain. By selecting individual single colony as described previously [16, 17], the cultures from an iridescent smooth colony [16, 17] which consisted of encapsulated organisms, were used for the preparations of agglutinogen and bacterin.

Chickens: Eggs from grandparent stock Heisdorf and Nelson (Shizuoka-Ken, Japan) known to be free from *Haemophilus* organisms and from certain viral and bacterial infections were incubated at our laboratory. The hatched chicks were used for the experiment.

Bacterins: The cultures incubated in S broth medium [7] at 37°C for 16 hr, were centrifuged at 8,000 g at 4°C for 30 min, washed once in phosphate-buffered saline (PBS, pH 7.2), resuspended in PBS containing 0.01% thimerosal, and adjusted to 5×10^9 cells/m*l* and 5×10^{10} cells/m*l* by spectrophotometer (Coleman Junior II, model 6/20, 650 nm, 5×10^{10} cells/m*l*=OD 0.386). Finally these were potenciated by adding $Al(OH)_3$ in 900 μg/m*l*.

Inoculation and challenge-exposure: Chickens of the first group were inoculated intramuscularly with 0.5 m*l* or 1 m*l* of bacterin, a single dose at 4 weeks of age, the second group with double doses at 4 and 7 weeks of age, or at 5 and 13 weeks of age, and the third group with triple doses at 4, 7

and 10 weeks of age. At 3, 5 or 17 weeks after the final inoculation, the chickens were intranasally challenged with the live organisms of iridescent type [16, 17] at least 10^8 colony-forming units/0.2 ml/chicken, and were daily observed clinical signs, especially nasal discharge and facial swelling. *Haemophilus* isolation was performed on day seven after the challenge-exposure. Chickens with coryza signs or/and with positive *Haemophilus* isolation were regarded as infection. Just before challenge-exposure, sera were obtained for the antibody titration.

Antibody determination: The non-treated antigens (NT) were prepared from strains 221 and H-18 by the method described previously [17]. The rapid plate agglutination (RPA) method was employed, and the results were read as the criteria described previously [7]. By using the hemagglutinating antigen prepared from strain 221 (iridescent type), hemagglutination-inhibition (HI) antibody was determined by the method described by Kato [5].

Results

Cross-protection tests among the bacterins prepared from strains 221 and H-18, and various isolates of serotypes 1 and 2: Protection tests were performed in chickens between bacterins prepared from strains 221 and H-18 by the intranasal challenge-exposure with various isolates of serotypes 1 and 2 (Table 1). Both HI antibody and agglutinin against 221-NT (anti-221-NT) were found in 90% to 100% of the chickens inoculated with 221 bacterin, which protected in 70% to 90% against the challenge-

Table 1. Protective activity of bacterins* prepared from *Haemophilus paragallinarum* strains 221 or H-18 against intranasal challenge-exposure of various strains of serotypes 1 and 2

Inoculated (5×10^9 cells/ml) with (Serotype)	Challenge-exposed**		Serum antibody when challenge-exposed				Protective activity				No. of chickens with coryza signs/No. of unvaccinated control chickens used
	Strain (Serotype)	No. of chickens	No. of HI positive (≧5) chickens	Geometric mean of HI titer	No. of RPA positive (≧5) chickens against 221-NT	No. of RPA positive (≧5) chickens against H-18-NT	No. of chickens with coryza signs***	No. of chickens with *Haemophilus* isolation	No. of chickens infected	Protective rate (%)	
221 (1)	221 (1)	10	10	86.5	10	0	1	2	2	80	5/5
	7682 (1)	10	10	72.8	10	0	2	2	2	80	5/5
	7119 (1)	10	10	72.4	10	0	2	3	3	70	5/5
	7411 (1)	10	9	51.8	10	0	2	3	3	70	5/5
	7161 (1)	10	10	82.3	10	0	1	2	2	80	5/5
	7301 (1)	10	10	96.8	10	0	0	1	1	90	5/5
	H-18 (2)	10	10	88.5	10	0	10	10	10	0	5/5
	1101 (2)	10	9	93.4	9	0	10	10	10	0	5/5
	7710 (2)	10	9	68.7	10	0	10	10	10	0	5/5
	FO-8 (2)	10	10	72.6	10	0	10	10	10	0	5/5
H-18 (2)	221 (1)	10	0	<2.5	0	4	10	10	10	0	5/5
	7682 (1)	10	0	<2.5	0	3	10	10	10	0	5/5
	7119 (1)	10	0	<2.5	0	3	10	10	10	0	5/5
	7411 (1)	10	0	<2.5	0	4	10	10	10	0	5/5
	7161 (1)	10	0	<2.5	0	4	10	10	10	0	5/5
	7301 (1)	10	0	<2.5	0	3	10	10	10	0	5/5
	H-18 (2)	10	0	<2.5	0	4	2	3	3	70	5/5
	1101 (2)	10	0	<2.5	0	4	5	6	6	40	5/5
	7710 (2)	10	0	<2.5	0	3	2	1	2	80	5/5
	FO-8 (2)	10	0	<2.5	0	4	2	3	3	70	5/5

* Inoculated (1 ml/chicken) at 4 weeks of age, challenge-exposed at 7 weeks of age, and sacrificed at 8 weeks of age.

** Challenge-exposure doses (at least 10^8 colony-forming units/0.2 ml/chicken).

*** Nasal discharge and/or facial swelling.

HI, Hemagglutination-inhibition; RPA, Rapid plate agglutination; NT, Non-treated agglutinogen.

Table 2. Protective activity of bacterins* prepared from *H. paragallinarum* strains of serotypes 1 and 2 against homologous and heterologous challenge-exposure

Inoculated (5×10^9 cells/m*l*) with (Serotype)	Challenge-exposed**		Serum antibody when challenge-exposed				Protective activity			
	Strain (Serotype)	No. of chickens	No. of HI positive (≧5) chickens	Geometric mean of HI titer	No. of RPA positive (≧5) chickens against 221–NT	No. of RPA positive (≧5) chickens against H–18–NT	No. of chickens with coryza signs	No. of chickens with *Haemophilus* isolation	No. of chickens infected	Protective rate (%)
221 (1)		10	10	68.5	10	0	1	2	2	80
7682 (1)		10	10	72.5	10	0	0	2	2	80
7119 (1)	221 (1)	10	10	66.7	9	0	2	3	3	70
7411 (1)		10	9	52.3	10	0	2	2	3	70
7161 (1)		10	10	44.8	10	0	1	2	2	80
7301 (1)		10	10	74.5	10	0	2	2	2	80
H–18 (2)		10	0	<2.5	0	4	2	3	3	70
1101 (2)		10	0	<2.5	0	3	4	6	6	40
7710 (2)	H–18 (2)	10	0	<2.5	0	5	2	3	3	70
FO–3 (2)		10	0	<2.5	0	2	2	3	4	60
K–6 (2)		10	0	<2.5	0	1	4	5	5	50
FO–26 (2)		10	0	<2.5	0	3	3	4	4	60
Control	221 (1)	5	0	<2.5	0	0	5	5	5	—
	H–18 (2)	5	0	<2.5	0	0	5	5	5	—

* See, Table 1.

** Challenge-exposure doses (221=2.6×10^8 colony-forming units/0.2 m*l*/chicken, H–18=4.5×10^8).
HI, Hemagglutination-inhibition; RPA, Rapid plate agglutination; NT, Non-treated agglutinogen.
No protection was observed in chickens challenge-exposed with heterologous strains.

exposure with the isolates of serotype 1. Agglutinin against H-18-NT (anti-H-18-NT) was also detected in 30% to 40% of the chickens inoculated with H-18 bacterin, which protected in 40% to 80% against challenge-exposure with the isolates of serotype 2. All the chickens in the heterologous challenge-exposure group were not survived.

Chickens inoculated with the bacterins prepared from various isolates of serotypes 1 and 2 were challenged with strains 221 and H-18 (Table 2). The chickens inoculated with the bacterins prepared from the serotype 1 strains produced in 90% to 100% both HI antibody and anti-221-NT agglutinin, and protected in 70% to 80% against challenge-exposure with strain 221. Ten to 50% of the chickens inoculated with the bacterins prepared from the serotype 2 strains produced anti-H-18-NT agglutinin, and 40% to 70% of the chickens protected against challenge-exposure with strain H-18. All the chickens in the heterologous challenge-exposure groups were not survived.

Cross-immunization tests between serotypes 1 and 2 were performed in the chickens inoculated with triple doses (Table 3). HI antibody was detected in the chickens inoculated with 221 bacterin but not in the chickens with H-18 bacterin. Of 40 sera obtained from 221 and H-18 inoculated group chickens, 35 reacted to homologous agglutinogen, but 5 showed slight agglutination against heterologous agglutinogen. No protection against challenge-exposure with heterologous was observed in such a highly immunized chickens.

Immunization with the 221 bacterins in varing concentrations: Even in the chickens given a large amount of antigen, protection

Table 3. Cross-immunization test* between serotypes 1 and 2 of *H. paragallinarum* (triple doses)

Inoculated (5×10^9 cells/m*l*) with (Serotype)	Challenge-exposed**		Serum antibody when challenge-exposed				Protective activity			
	Strain (Serotype)	No. of chickens	No. of HI positive (≧5) chickens	Geometric mean of HI titer	No. of RPA positive (≧5) chickens against 221-NT	No. of RPA positive (≧5) chickens against H-18-NT	No. of chickens with coryza signs	No. of chickens with *Haemophilus* isolation	No. of chickens infected	Protective rate (%)
221 (1)	221 (1)	10	10	24.2	10	1	0	1	1	90
	H-18 (2)	10	10	30.2	10	1	9	10	10	0
H-18 (2)	221 (1)	10	0	<2.5	2	9	10	10	10	0
	H-18 (2)	10	0	<2.5	1	8	0	1	1	90
Control	221 (1)	5	0	<2.5	0	0	5	5	5	—
	H-18 (2)	5	0	<2.5	0	0	5	5	5	—

* Inoculated (1 m*l*/chicken) at 4, 7 and 10 weeks of age, challenge-exposed at 13 weeks of age, and sacrificed at 14 weeks of age.

** Challenge-exposure doses (221=2.5×10^8 colony-forming units/0.2 m*l*/chicken, H-18=3.5×10^8).
HI, Hemagglutination-inhibition; RPA, Rapid plate agglutination; NT, Non-treated agglutinogen.

Table 4. Efficacy of bacterins* prepared from *H. paragallinarum* strain 221 of serotye 1 in varying concentrations

Bacterin concentration (Cells/m*l*)	Challenge-exposed**		Serum antibody when challenge-exposed				Protective activity			
	Strain (Serotype)	No. of chickens	No. of HI positive (≧5) chickens	Geometric mean of HI titer	No. of RPA positive (≧5) chickens against 221-NT	No. of RPA positive (≧5) chickens against H-18-NT	No. of chickens with coryza signs	No. of chickens with *Haemophilus* isolation	No. of chickens infected	Protective rate (%)
5×10^{10}	221 (1)	10	9	33.8	9	0	1	1	1	90
	H-18 (2)	10	9	48.7	9	0	9	10	10	0
1×10^{10}	221 (1)	10	10	110.0	10	0	1	2	2	80
	H-18 (2)	10	10	154.2	9	0	10	10	10	0
2.5×10^9	221 (1)	10	10	204.1	10	0	1	1	1	90
	H-18 (2)	10	10	183.2	10	0	8	9	10	0
Control	221 (1)	5	0	<2.5	0	0	5	5	5	—
	H-18 (2)	5	0	<2.5	0	0	5	5	5	—

* See, Table 1.

** Challenge-exposure doses (221=6×10^8 colony-forming units/0.2 m*l*/chicken, H-18=1.2×10^8).
HI, Hemagglutination-inhibition; RPA, Rapid plate agglutination; NT, non-treated agglutinogen.

was only found in the homologous challenge-exposure groups (Table 4). Geometric mean of HI antibody titer in the chickens given the 5×10^{10} bacterin was significantly lower than that of the 1×10^{10} or 2.5×10^9 bacterins.

Immunization with the H-18 bacterin: In the single dose of the bacterins, 25% to 30% of the chickens inoculated with 0.5 m*l* produced anti-H-18-NT agglutinin, while 35% to 40% of the chickens with 1 m*l* did. The former protected in 60% against challenge-exposure with strain H-18, and the latter in 65% to 70% (Table 5). In the double doses of the bacterins, 85% to 90% of the chickens inoculated with 0.5 m*l* and 1 m*l* produced anti-H-18-NT agglutinin, while 90% of the chickens with 1 m*l* and 0.5 m*l* did. The former protected in 80%

Table 5. Efficacy of the bacterins prepared from *H. paragallinarum* strain H-18 against challenge-exposed with strain H-18

Bacterin (5×10^9 cells/ml) Lot No.	Inoculation at: First (4 weeks of age)	Inoculation at: Second (7 weeks of age)	Challenge-exposed*: Weeks of age	Challenge-exposed*: No. of chickens	Serum antibody when challenge-exposed: Geometric mean of HI titer	Serum antibody when challenge-exposed: No. of RPA positive (≧5) chickens against 221-NT	Serum antibody when challenge-exposed: No. of RPA positive (≧5) chickens against H-18-NT	Protective activity: No. of chickens with coryza signs	Protective activity: No. of chickens with *Haemophilus* isolation	Protective activity: No. of chickens infected	Protective activity: Protective rate (%)
1	0.5**	—	7	20	<2.5	0	6	8	8	8	60
	1.0	—	7	20	<2.5	0	8	6	6	6	70
	—	—	7	5	<2.5	0	0	5	5	5	—
	0.5	1.0	10	20	<2.5	0	18	2	4	4	80
	1.0	0.5	10	20	<2.5	0	18	0	2	2	90
	—	—	10	5	<2.5	0	0	5	5	5	—
2	0.5	—	7	20	<2.5	0	5	7	8	8	60
	1.0	—	7	20	<2.5	0	7	5	7	7	65
	—	—	7	5	<2.5	0	0	5	5	5	—
	0.5	1.0	10	20	<2.5	0	17	3	4	4	80
	1.0	0.5	10	20	<2.5	0	18	3	3	3	85
	—	—	10	5	<2.5	0	0	5	5	5	—

* Challenge-exposure doses (single dose=8.8×10^8 colony-forming units/0.2 ml/chicken, double doses=7.6×10^8).
** Dosage (ml/chicken).
HI, Hemagglutination-inhibition; RPA, Rapid plate agglutination; NT, Non-treated agglutinogen.

Table 6. Durability of immunity in chickens inoculated with bacterin prepared from *H. paragallinarum* strain H-18

Inoculation (5×10^9 cells/ml) at: First (5 weeks of age)	Inoculation at: Second (15 weeks of age)	No. of chickens challenge-exposed*	No. of chickens infected and protective rate (%) against challenge-exposed at: 8 weeks of age	15 weeks of age	18 weeks of age	25 weeks of age	30 weeks of age	45 weeks of age
0.5**	1.0	10	3 (70)	4 (60)	3 (70)	3 (70)	3 (70)	4 (60)
1.0	0.5	10	2 (80)	2 (80)	1 (90)	1 (90)	2 (80)	2 (80)
—	—	5	5 (—)	5 (—)	5 (—)	5 (—)	5 (—)	5 (—)

* Chickens were challenge-exposed with strain H-18 with at least 10^8 colony-forming units/0.2 ml/chicken.
** Dosage (ml/chicken).

against challenge-exposure with strain H-18, and the latter in 85% to 90%.

Durability of immunity in the chickens inoculated with the H-18 bacterin was investigated (Table 6). Eighty to 90% of the chickens provided double doses of the bacterin 1 ml and 0.5 ml, protected against challenge-exposure with strain H-18, while 60% to 70% of the chickens with 0.5 ml and 1 ml did. The protective immunity still persisted in significant level at 30 weeks after the postinoculation with double doses of the bacterin.

Discussion

In the present paper, to clarify the immunologic relationship between Page's serotype strains and Sawata's ones, im-

munogenicity of fresh isolates of *H. paragallinarum* Sawata's serotypes 1 and 2 [16] was studied. Eleven isolates and strain 221 of serotype 1 [6] were divided into two immunotypes by cross-protection test. Of these five strains of serotype 1, 7682, 7719, 7411, 7161 and 7301 as well as strain 221 these five strains of serotype 1, 7682, 7719, had similar immunogenicity and belonged to one immunotype (1), while six strains of serotype 2, H-18, 1101, 7710, FO-8, K-6 and FO-26 belonged to the other immunotype (2). No cross-protection was observed between these immunotypes, even in the hyper-immunized chickens which inoculated with concentrated becterins or with triple doses. Using the isolates other than strains 221 and H-18, it is confirmed that serotype specificity of the strains is closely correlated to immunospecificity.

A discrepancy exists in the reports on the immunospecificity of *H. paragallinarum*. Page et al. [13] found the presence of cross-protection among their three serotypes A, B and C. However, Matsumoto and Yamamoto [10] found no cross-protection between monovalent bacterins of strain Modesto and strain 17756, both considered to belong to Page's serotype A. Davis et al. [1, 2] also had reported the lack of cross-protection within serotype A strains W and Modesto which was classified to serotype C by Rimler et al. [14], recently to Sawata's serotype 2 by the present authors [18].

Rimler et al. [14, 15] described the existance of three immunotypes among their strains, but, we have so far confirmed two immunotypes among their strains [9]. Previously, we [16, 17] reported that the encapsulated organisms forming iridescent colony were divided into serotypes 1 and 2, and that Page's serotype B strains 0222 and Spross used were untypable, because of lack of serotype B-specific antigen [18]. Further experiments are necessary to clarify whether the other serotype or imunotype such as Page's serotype (immunotype) B exists.

Kato [4] described that there was cross-protection among Kato and Tsubahara's types I, II and III [6]. However, as previously demonstrated [16], both their types II and III were variants derived from their type I strain.

Rimler et al. [14, 15] reported that presence of cross-protection among their immunotypes, and that a shared antigen was responsible for protection. However, our previous data [8, 17] obtained from antigenic analysis of both strains 221 and H-18, indicated that serotype-specific antigen itself seemed to be protective antigen. The discrepancy between Rimler et al. [14, 15] and our observations might be due to differences in the quality of bacterin, inoculation method, challenge strain, challenge dose and the criteria for judging immunity and so on.

The bacterins prepared from serotype 1 strains produced in birds both HI antibody and anti-221-NT agglutinin. Kato [5] and Otsuki and Iritani [11] described that HI antibody was significantly correlated with the protective activity against infection with serotype 1. Iritani et al. [3] reported that HI test was one of the useful methods for evaluating potency of the bacterin. In the present data, a correlation was observed between HI antibody or anti-221-NT agglutinin titers and protective activity. As far as serotype 1 bacterin, HI test seems to be one of the simple and useful method for evaluating protective potency of the bacterin. However, all the serotype 2 strains never produced HI antibody, but protected enough against homologous challenge-exposure. It is also of interest whether hem-agglutinating substance and HI antibody are essentially responsible for the mecha-

nisms of infection in the mucous membrane and of its protection.

For evaluating efficacy of the bacterins, clinical coryza signs were generally employed [1, 2, 11, 13–15]. Culture method from challenge-exposed chickens was also employed by Matsumoto and Yamamoto [10], though their recovery rate was significantly lower than those of morbidity. In the present experiment, the inoculated organisms for challenge infection were constantly recovered from the chickens with clinical coryza signs, and the recovery rate was rather higher than those of morbidity. The uses of improved identification method and media may cause these. We may take the culture method as a useful technique for evaluating potency of the bacterin.

The present investigation showed that the chickens inoculated with serotype 2 bacterin, even in a single dose, produced the protection against homologous challenge-exposure strains, though no HI antibody and significant individual variation in agglutinin production ($P<0.01$) was produced even in the groups inoculated with double doses. This shows that antigenicity to produce agglutinin in serotype 2 strains might be lower than that of serotype 1 strains.

H. paragallinarum H-18 bacterin exhibited an overall average protection rate of 63.5% with a single dose (0.5 m*l* dose:60%, 1 m*l* dose:67.5%) and 83.5% with double doses (0.5 m*l*+1 m*l* dose:80%, 1 m*l*+0.5 m*l* dose:87.5%) against strain H-18. In the type 2 bacterin, there is a tendency that use of larger dosage in the primary inoculation improves the protection rate.

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要　約

鶏のヘモフイルス感染症に関する研究　3. ヘモフイルス・パラガリナルム血清型1及び2の菌株の免疫原性：久米勝巳・澤田　章・中瀬安清（北里研究所）——ヘモフイルス・パラガリナルム澤田の血清型1及び2に属する分離株のバクテリンについて免疫原性を調べた．血清型1の221株を対照とし，両型11株の新分離株は2つの免疫型に分かれ，明かに型特異性の免疫を示し，頻回接種や免疫菌量の増量でも両型間の交差免疫性は全く認められなかった．また，この免疫型は血清型特異性と密接に関連することが確認された．血清型1のバクテリンではどの株も血球凝集抑制（HI）抗体の産生を認めたが，高度に濃縮されたバククテリンを接種された鶏では HI 抗体産生が抑制された．血清型2のバクテリンではどの株も HI 抗体の産生はなく，1回注射で充分な防御免疫が得られたが，凝集素産生能は鶏個体差による変動が著るしく，また1回注射群と2回注射群における凝集素産生能に有意差が認められたことから，血清型2の各株とも血清型1の各株に比べ，凝集原としての抗原性が低いことが示唆された．