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Proposal of a New Serovar and Altered Nomenclature for *Haemophilus paragallinarum* in the Kume Hemagglutinin Scheme

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By using the Kume hemagglutinin serotyping scheme, 13 Australian isolates of *Haemophilus paragallinarum* were shown to constitute a new serovar within the presently termed serogroup II. Because of the likelihood that new serovars will continue to be established, we propose a rationalization of the nomenclature of the Kume scheme. Under this altered scheme, the three recognized serogroups I, II, and III are renamed A, C, and B, respectively. Within each of the serogroups, the serovars are numbered sequentially, allowing new serovars to be added in numerical order. Thus, the nine currently recognized Kume serovars are termed A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-4.

Haemophilus paragallinarum is the causative agent of infectious coryza, an economically important upper respiratory tract disease of chickens (17). The major economic effect of the disease is an increased culling rate in meat chickens and a reduction in egg production (10 to 40%) by laying and breeding hens, particularly on multiage farms (17).

The first serological classification of *H. paragallinarum* was performed in the United States by Page (12), who used a plate agglutination test to recognize three serovars (A, B, and C). Subsequently, the Page scheme has been used in both Germany (4) and Australia (1, 16). In independent studies, groups in Japan also used agglutination tests to recognize two or three agglutinin serovars (7, 15).

Two other serotyping schemes have been reported—the Hinz scheme based on heat-stable antigens detected in a gel diffusion test (5) and the Kume scheme based on hemagglutinating antigens obtained by extraction with potassium thiocyanate followed by sonication (11). Originally, the Kume scheme recognized three serogroups, termed I, II, and III, and a total of seven serovars, termed HA-1 to HA-7 (11). We have recently recognized an eighth serovar (3).

In this paper, we report the existence of a ninth hemagglutinin serovar within the Kume scheme. In addition, we propose an altered nomenclature for the Kume scheme, which indicates the close linkage of this scheme with the Page scheme, allows a logical method of adding new serovars, and emphasizes the relationships between the serovars.

MATERIALS AND METHODS

Cultures. The eight reference serovar cultures of the Kume scheme used were 221 (HA-1), 2403 (HA-2), E-3C (HA-3), H-18 (HA-4), Modesto (HA-5), SA-3 (HA-6), 2671 (HA-7), and HP14 (HA-8). A total of 13 Australian field isolated of *H. paragallinarum* were examined. The 13 isolates were obtained from three properties (farms A, B, and C) located in southeast Queensland, Australia. Farms A and B, from which seven of the isolates were obtained, had common ownership, and birds were exchanged between the two properties. Farm C, from which the remaining isolates were obtained, was located over 180 kilometers from both farms A and B. No factor such as ownership or bird exchange could be identified to link farm C with farms A and B.

Medium. Test medium agar (13) supplemented with 5% (vol/vol) oleic albumin complex, 1% (vol/vol) filter-sterilized and heat-inactivated chicken serum, and 0.00025% (wt/vol) NAD was used. This medium was termed TM/SN.

Antisera. The rabbit antisera for serovars HA-1 to HA-8 used in this study were those produced in a previous study (3). Rabbit antiserum to one of the Australian field isolates (HP60) was produced in a similar manner. Briefly, bacterial cells grown overnight on TM/SN were harvested into 0.01 M phosphate-buffered saline (PBS; pH 7.0), washed once, resuspended in PBS containing 0.01% (wt/vol) thimerosal, and adjusted to an optical density of 1.6 at 650 nm on a spectrophotometer (model 2600; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). A portion (1 ml) of a suspension containing equal volumes of the adjusted antigen and Freund complete adjuvant was inoculated subcutaneously into rabbits on two occasions with an interval of 3 days. Subsequent injections were given intravenously at 3-day intervals and consisted of 0.5, 0.5, 1, 1, 2, 2, 4, and 4 ml of the adjusted antigen. The rabbits were exsanguinated 7 days after the last inoculation.

Hemagglutinins. Hemagglutinins were prepared as described previously (3). Briefly, cultures were grown overnight on TM/SN, harvested into PBS, washed once in PBS, suspended in 0.5 M KSCN-0.425 M NaCl (pH 6.3), adjusted to an optical density of 1.6 at 650 nm, stirred for 2 h at 4°C, and sonicated (30 s; 60% pulsed output; power output, 5) (model W375; Ultrasonics) while being held on ice. The antigen was washed three times in PBS and suspended in PBS with 0.01% (wt/vol) thimerosal to an optical density of 1.6 at 650 nm (Gilford 2600).

Chicken erythrocytes. Glutaraldehyde-fixed chicken erythrocytes were prepared as described previously (3). Briefly, chicken blood was collected into Alsevers solution, the erythrocytes were harvested by centrifugation and washed three times in 0.15 M NaCl, and a 1% solution of erythrocytes in a glutaraldehyde-salt solution (1% glutaraldehyde in 0.01 M $NaPO_4$, pH 8.2, 0.01 M NaCl) was prepared and held at 4°C for 30 min. The glutaraldehyde-fixed erythrocytes were collected by centrifugation, washed five times in 0.15 M NaCl and five times in distilled water, and held at a 30%

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TABLE 1. HI antibody titers of isolate HP60 antiserum to hemagglutinins from isolates H-18, Modesto, SA-3, and HP60, both before and after absorption

HP60 antiserum	HI titer[a] with hemagglutinin from (serovar):			
	H-18 (HA-4)	Modesto (HA-5)	SA-3 (HA-6)	HP60 (HA-9)
Unabsorbed	40,960	40,960	80	40,960
Absorbed[b]	0	0	0	10,240

[a] Zero indicates a negative HI test at a serum dilution of 1:40.
[b] Absorbed twice each with hemagglutinin from H-18 and Modesto.

concentration in distilled water containing 0.01% thimerosal. Before use, the glutaraldehyde-fixed erythrocytes were suspended to 1% in PBS with 0.1% (wt/vol) bovine serum albumin and 0.001% (wt/vol) gelatin.

Hemagglutination and hemagglutination inhibition tests. The hemagglutinating titers of the extracted and sonicated antigens were determined as previously described (3). The hemagglutination inhibition (HI) test was performed as described previously by using four hemagglutinin units (3).

Antiserum adsorption. Specific antiserum to HP60 was produced by two adsorptions each with hemagglutinin prepared from isolates Modesto and H-18 by using the technique previously described (3).

Serotyping. The Australian isolates were assigned to a Kume serogroup by performing HI tests with unabsorbed antisera to all eight recognized serovars. Assignment to a serovar was made by performing HI tests using specific absorbed sera for those serovars positive in the serogrouping test.

RESULTS

Hemagglutinin serotyping. The 13 Australian isolates were identified as belonging to Kume serogroup II since they failed to react with any of the five antisera of serogroups I and III but did react with all three unabsorbed antisera of serogroup II at a dilution of 1:160 or higher. However, only low titers could be detected with the three specific absorbed antisera for serogroup II (H-18, Modesto, and SA-3). One of the Australian isolates (HP60) was selected, and rabbit antiserum was prepared. The unabsorbed antisera to HP60 exhibited HI activity against isolates H-18, Modesto, and SA-3 (serogroup II) but not against isolates 221, 2403, E-3C, HP14, or 2671 (serogroups I and III). The cross-reactions to isolates H-18, Modesto, and SA-3 were removed by absorption, leaving only a HI titer to HP60. The HI titers of HP60 antiserum to the homologous hemagglutinin as well as to the other representative hemagglutinins of serogroup II, both before and after absorption, are shown in Table 1. By using the absorbed HP60 antiserum, it was shown that the remaining 12 Australian isolates belonged to the same new hemagglutinin serovar, with HP60 as the serovar reference isolate. Under the existing nomenclature of the Kume scheme, the new serovar would be placed in serogroup II and termed HA-9.

Proposal for altered Kume serotyping nomenclature. We propose to alter the nomenclature of the Kume serotyping scheme such that serogroups I, II, and III are renamed A, C, and B, respectively. In addition, the original serovars which were identified as HA-1 to HA-8 are renamed numerically within each serogroup. Table 2 illustrates the original nomenclature and the proposed new nomenclature.

TABLE 2. Comparison of original and newly proposed nomenclature for the Kume serotyping scheme for *H. paragallinarum*

Reference isolate	Original scheme		New scheme	
	Serogroup	Serovar	Serogroup	Serovar
221	I	HA-1	A	A-1
2403	I	HA-2	A	A-2
E-3C	I	HA-3	A	A-3
HP14	I	HA-8	A	A-4
H-18	II	HA-4	C	C-1
Modesto	II	HA-5	C	C-2
SA-3	II	HA-6	C	C-3
HP60	II	HA-9	C	C-4
2671	III	HA-7	B	B-1

DISCUSSION

In their original study of a hemagglutinin-serotyping scheme for *H. paragallinarum*, Kume et al. (11) recognized three serogroups which could be split into seven serovars by using absorbed antisera. Subsequently, we have recognized two new serovars, HA-8, a member of original serogroup I (3), and now, a ninth serovar, this one being in original serogroup II.

These two recently recognized serovars have disrupted the logical assignment of serogroups and serovars as proposed by Kume et al. (11) (Table 2). Since it seems likely that other serovars will be recognized, this problem of illogical nomenclature will increase. Further, as the number of serovars increases, it will no longer be possible for workers to immediately recognize the serogroup to which a particular serovar belongs. It is not logical that serovars HA-1, HA-2, HA-3, and HA-8 belong to serogroup I, while serovars HA-4, HA-5, HA-6, and HA-9 belong to serogroup II. The proposed altered nomenclature will remove this problem. Kume serovars HA-1, HA-2, HA-3, and HA-8 would be termed A-1, A-2, A-3, and A-4 and would all be instantly recognized as belonging to Kume serogroup A (formerly serogroup I).

The new nomenclature system allows quicker recognition of the serogroup. The majority of the over 100 isolates of *H. paragallinarum* that have been examined by both the Kume and Page schemes have shown a correlation between the results of the two schemes (3, 11). With the possible exception of two isolates examined by Kume et al. (11), all Page serovar A, B, and C isolates would belong to the new Kume serogroups A, B, and C. The new nomenclature for the Kume scheme thus emphasizes the close linkage between the well-established Page serovars and the Kume serogroups.

The possible exceptions to the correlation between the Page and Kume schemes are isolates 2403 and 1596. These exceptions may be more apparent than real, since there is some dispute over the Page serovar to which these isolates belong. According to Hinz and Kunjara (6), both isolates belong to Page serovar B. However, isolate 2403 is listed in *Bergey's Manual of Systematic Bacteriology* (8) as Page serovar A. Our results (3) support the latter finding. The overall evidence is that isolate 2403 indeed belongs to Page serovar A, a conclusion in accordance with the finding that isolate 2403 belongs to Kume serogroup A (formerly serogroup I) (3, 11). Isolate 1596, which belongs to Kume serogroup A (11), needs to be reexamined by using the Page scheme.

Several studies have suggested a correlation between the

Page serovar and type-specific immunity, since vaccines based on Page serovar A organisms protect against a serovar A challenge but not a serovar C challenge (9, 14). However, these studies, limited to a few isolates, were performed before the development of the Kume scheme allowed the recognition that isolates of the same Page serovar possessed different antigens. As an example, *H. paragallinarum* isolates H-18 and Modesto belong to Page serovar C but belong to Kume serovars C-1 and C-2, respectively (11). To date, it has been established only that isolates H-18 and Modesto (Kume serovars C-1 and C-2) are cross protective (10) and that isolate 21 (Kume serovar A-1) does not provide cross protection against either H-18 or Modesto (Kume serovars C-1 and C-2) (10). Further studies to establish the immunological relationships among the Kume serovars are required.

Such cross protection studies are of practical importance. For example, we have shown the presence, in Australia, of three Kume serovars—A-4 and C-2 (3) and C-4 (Table 1). Since Kume serovars A-4 and C-2 are not cross protective (2), commercial vaccines in Australia include both serovars. It remains to be determined whether a serovar C-4 isolate should be included in such a vaccine or whether a serovar C-2 isolate provides adequate cross protection.

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