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Haemophilus avium, a New Species from Chickens

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Twelve *Haemophilus* strains (previously designated group II strains by Hinz) from chickens and of uncertain taxonomic position and 29 *Haemophilus paragallinarum* strains were investigated. The findings indicate the existence of a hitherto unknown species, for which the name *Haemophilus avium* sp. nov. is proposed. The main characters which differentiate *H. avium* from *H. paragallinarum* are as follows: all of the *H. avium* but none of the *H. paragallinarum* strains tested produce catalase, alkaline phosphatase, and α-glucosidase, acidify galactose and trehalose, and do not require serum for optimal growth. Most of the *H. avium* strains produce yellow pigment, grow aerobically, and do not require CO_2 for optimal growth. In further contrast to *H. paragallinarum*, none of the *H. avium* strains are able to cause infectious coryza of chickens. The type strain of *H. avium* is IPDH 2654 (= ATCC 29546).

De Blieck (5), Nelson (20), and Elliot and Lewis (8) were the first to report on the etiology of the specific disease of chickens known as *Coryza infectiosa gallinarum*. The name now used for this disease, infectious coryza, was introduced by Schalm and Beach (26). Infectious coryza is world-wide in distribution and is of economic importance wherever chickens are raised. De Blieck named the causative agent *Bacillus haemoglobinophilus coryzae gallinarum*, and later Delaplane et al. (6) named it *Haemophilus gallinarum*, by which name it is currently cited in *Bergey's Manual of Determinative Bacteriology* (23). Schalm and Beach (25, 26) and Delaplane et al. (7) found that their strains required X and V factors for growth. Later, this finding could not be confirmed by other workers (1, 9, 21), who showed that the coryza-producing agent requires only V factor for growth. Biberstein and White (2), therefore, proposed the name *Haemophilus paragallinarum* for X-factor-independent haemophili which are able to cause infectious coryza of chickens. In *Bergey's Manual* (30) and in Cowan and Steel's manual (4), *H. paragallinarum* was included in the genus *Haemophilus* as a recognized species.

Furthermore, there are X-factor-independent haemophili in chickens which differ from *H. paragallinarum* in their pathogenicity (they are unable to produce infectious coryza) and in their physiological, biochemical and serological features. To date, these haemophili have been unnamed and unclassified (9, 16, 21, 24). Consequently, a study was undertaken to determine the taxonomic relationship of these organisms, and the results of this study are presented herein.

MATERIALS AND METHODS

Bacterial strains. The designations and sources of the strains used in this study are listed in Table 1. Cultures of the strains were stored in the freeze-dried state until needed.

Morphological, physiological, and biochemical tests. The morphologies of cells and colonies and the staining reactions (Gram and capsule staining) were determined as described by Hinz (9).

The morphologies and the requirement for V and X factors were determined by use of the following basic media: (i) proteose peptone agar consisted of 2.0% (wt/vol) proteose peptone no. 3 (Difco), 0.6% (wt/vol) NaCl, 0.5% (wt/vol) glucose, and 1.0% (wt/vol) Noble agar; the final pH was 7.2 to 7.3; (ii) tryptose agar (Difco); the final pH was 7.2 to 7.3; (iii) brain heart infusion agar (BHIA) (Difco); the final pH was 7.2 to 7.3.

The media were adjusted to pH 7.6 with 1 M NaOH and were then autoclaved for 10 min. X factor and serum (X, S), or X and V factors (X, V), or V factor (V) or V factor and serum (V, S) were added to each medium. A filter-sterilized solution of β-nicotinamide adenine dinucleotide (NAD) (Serva) and cysteine-hydrochloride (Merck) was added to give a final concentration of 100 μg/ml for both substances. Hemin (Roth) dissolved in triethanolamine (Kodak) was added as a filtered solution to give a final concentration of 10 μg/ml of medium. The chicken sera added to the media were free of agglutinating antibodies against the *Haemophilus* strains used. Inoculated media were incubated aerobically, microaerophilically (90% air and 10% CO_2 [vol/vol]), and anaerobically (BBL-Gas Pak 110 System) for 24 and 48 h at 37°C. The results were evaluated as described by Zinnemann et al. (31). *Haemophilus influenzae* strain TS 43 was used as a control.

The ability to synthesize porphyrin from δ-aminolevulinic acid was determined as described by Kilian (15).

For satellite growth and hemolysis, 24- to 48-h-old

TABLE 1. *Strains utilized in this study*

Strain no. in this study	Name as received	Isolated from:	Source and strain designation[a]
TS1	*Haemophilus* sp.	Chicken, air sac	IPDH 1762; serovar 1 (9-11)
TS2	*Haemophilus* sp.	Chicken, air sac	IPDH 64; serovar 1 (9-11)
TS3	*Haemophilus* sp.	Chicken, infraorbital sinus	IPDH 2654; serovar 2 (9-11)
TS4	*Haemophilus* sp.	Chicken, lung	IPDH 2659; serovar 2 (9-11)
TS5	*Haemophilus* sp.	Chicken, nose	IPDH 780; serovar 3 (9-11)
TS6	*Haemophilus* sp.	Chicken, infraorbital sinus	IPDH 94; serovar 4 (9-11)
TS7	*Haemophilus* sp.	Chicken, infraorbital sinus	IPDH 1254; (9-11)
TS8	*Haemophilus* sp.	Chicken, wattle	IPDH 0003 (Hinz)
TS9	*Haemophilus* sp.	Chicken, heart	IPDH 0002 (Hinz)
TS10	*Haemophilus* sp.	Chicken, infraorbital sinus	IPDH 280 (Hinz)
TS11	*Haemophilus* sp.	Chicken, infraorbital sinus	IPDH 331A (Hinz)
TS12	*Haemophilus* sp.	Chicken, infraorbital sinus	IPDH 306 (Hinz)
TS13	*H. gallinarum*		ATCC 14385 (=NCTC 3438)
TS14[a]	*H. gallinarum*	Chicken, respiratory tract	L. A. Page (21, 22); 0083; serovar A
TS15[a]	*H. gallinarum*	Chicken, respiratory tract	L. A. Page (21, 22); 0222; serovar B
TS16[a]	*H. gallinarum*	Chicken, respiratory tract	R. Yamamoto (27); 17756
TS17	*H. paragallinarum*	Chicken, sinus exudate	IPDH 1645; serovar A (9-11)
TS18	*H. paragallinarum*	Chicken, eye	IPDH 2213; serovar A (9, 10)
TS19	*H. paragallinarum*	Chicken, air-sac exudate	IPDH 1646; serovar A (9, 10)
TS20	*H. paragallinarum*	Chicken, sinus exudate	IPDH 1598; serovar A (9, 10)
TS21	*H. paragallinarum*	Chicken, sinus exudate	IPDH 6001; serovar A (9-11)
TS22	*H. paragallinarum*	Chicken, sinus exudate	IPDH 2403 (=ATCC 29545); serovar B (9-12)
TS23	*H. paragallinarum*	Chicken, sinus exudate	IPDH 2671; serovar B (9, 10)
TS24	*H. paragallinarum*	Chicken, sinus exudate	IPDH 1676; serovar B (9, 10)
TS25	*H. paragallinarum*	Chicken, sinus exudate	IPDH 2025; serovar B (9, 10)
TS26	*H. paragallinarum*	Chicken, sinus exudate	IPDH 2028; serovar B (9, 10)
TS27	*H. paragallinarum*	Chicken, sinus exudate	IPDH 733; serovar B (9, 11)
TS28	*H. paragallinarum*	Chicken, air-sac exudate	IPDH 2820; serovar B (9, 10)
TS29	*H. paragallinarum*	Chicken, nose exudate	IPDH 2600; serovar B (9, 10)
TS30	*H. paragallinarum*	Chicken, sinus exudate	IPDH 1655 (9, 10)
TS31	*H. paragallinarum*	Chicken, sinus exudate	IPDH 1596 (9, 10)
TS32	*H. paragallinarum*	Chicken, sinus exudate	IPDH 782 (9, 10)
TS33-42	*Haemophilus* sp.	Chickens, sinus exudate	IPDH, isolated recently (Hinz)
TS43	*H. influenzae*		HIM-417.7
TS44	*H. parainfluenzae*		ATCC 9796
TS45	*H. suis*		ATCC 19417
TS46	*H. parasuis*	Pig lung	G. Amtsberg; 3327
TS47	*Escherichia coli*		IPDH; field strain
TS48	*Yersinia pseudotuberculosis*		IPDH; field strain
TS49	*Staphylococcus epidermidis*		IPDH; field strain

[a] Strains received from R. Yamamoto, Department of Epidemiology and Preventive Medicine, School of Veterinary Medicine, University of California, Davis, Calif.

Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; IPDH, Institute for Poultry Diseases, Hannover, Germany; G. Amtsberg, Institute for Microbiology and Epidemic Animal Diseases, Hannover, Germany; HIM, Collection of the Hygiene-Institute, Marburg, Germany.

growth was examined on blood agar containing Columbia agar base (Oxoid) and 7% (wt/vol) defibrinated ox blood. A *Staphylococcus epidermidis* strain (TS 49) was used as a V-factor feeder.

Oxidase was tested for by Kovács's method (18).

Catalase activity was determined using tryptose agar-(X, V, S) and BHIA-(X, V, S) cultures which were incubated under microaerophilic and/or aerobic conditions. In testing for catalase, a few colonies were removed with a cover slip and mixed in a drop of 3% (vol/vol) H_2O_2. The production of bubbles was regarded as a positive test for catalase.

Indole production was demonstrated, using Kovács's reagent (4), in tryptose broth (Difco) with 50 μg of NAD per ml and 1% chicken serum after 1 and 3 days of growth.

Reduction of nitrate and nitrite was tested as described by Cowan (4) by use of the same tryptose broth as above but with 0.1% KNO_3.

The production of urease was determined as described by Lautrop (19).

Alkaline phosphatase was determined by the method of Kersters and DeLey (14).

Determination of the enzymes α-fucosidase and α-

glucosidase was performed by the method of Kilian and Bülow (17) and Kilian (16), using *p*-nitrophenyl-α-L-fucopyranoside and *p*-nitrophenyl-α-D-glucopyranoside (Serva) as substrates. *H. parainfluenzae* strain TS 44 and *Escherichia coli* strain TS 47 were used as positive controls for the α-glucoside test. One *Yersinia pseudotuberculosis* strain (TS 48), *H. parasuis* TS 46, and strain TS 45, listed as *H. suis*, acted as positive controls for the production of α-fucosidase.

Acid production from carbohydrates (Merck) was determined in phenol red broth base (Difco).

The base medium together with the carbohydrate (Table 2) and 1% (vol/vol) chicken serum was filter-sterilized through 0.2-μm-pore-size filters. The different test media were dispensed in 5-ml volumes in test tubes, and Durham tubes were inserted in the glucose medium for the detection of gas. The bacterial inoculum was added simultaneously with neutralized NAD

TABLE 2. *Characteristics of 13 previously unidentified strains of haemophili (includes ATCC 14385) and of 29 Haemophilus paragallinarum strains from chickens*[a]

Characteristic	Previously unidentified strains													*H. paragallinarum* strains
	TS13	TS3	TS4	TS1	TS2	TS5	TS6	TS7	TS8	TS9	TS10	TS11	TS12	TS14-TS42
X-factor requirement	−	−	−	−	−	−	−	−	−	−	−	−	−	− (0)[b]
δ-Aminolevulinic utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+ (29)
V-factor requirement	+	+	+	+	+	+	+	+	+	+	+	+	+	+ (29)
Aerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	− (0)
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+ (29)
CO_2 requirement	−	−	−	−	−	−	−	−	−	−	+[c]	+[c]	−	+ (29)
Serum requirement	−	−	−	−	−	−	−	−	−	−	−	−	−	−/+ (6)
Serum improves growth	−	−	−	−	−	−	−	−	−	−	−	−	−	+ (29)
Hemolysis	−	−	−	−	−	−	−	−	−	−	−	−	−	− (0)
Yellow-pigment production	+	−	+	+	+	+	+	+	+	+	+	+	+	− (0)
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+ (29)
Indole production	−	−	−	−	−	−	−	−	−	−	−	−	−	− (0)
Urease	−	−	−	−	−	−	−	−	−	−	−	−	−	− (0)
Oxidase	−	−	−	−	−	−	−	−	−	−	−	−	−	− (0)
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	− (0)
Alkaline phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	+	− (0)
ONPG[d]β-galactosidase	+	−	−	−	−	+	−	+	+	−	−	−	−	− (0)
α-Fucosidase	−	−	−	−	−	−	−	−	−	−	−	−	−	− (0)
PNPG[e]α-glucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+	−/+ (3)
Pathogenicity[f]	−	−	−	−	−	−	−	−	−	−	−	−	−	+ (29)
Acid from:														
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+ (29)[b]
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+ (29)
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+ (29)
Saccharose	+	+	+	+	+	+	+	+	+	+	+	+	+	+/− (27)
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	− (0)
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	− (0)
Mannitol	+	−	+	−	+	+	+	+	+	−	+	+	−	+ (29)
Sorbitol	−	−	−	−	−	−	−	+	+	−	−	−	−	+ (29)
Dextrin	+	+	+	+	+	+	+	+	+	−	+	+	+	+ (29)
Xylose	+	−	+	−	−	+	+	−	+	+	−	−	−	−/+ (6)
Lactose	−	−	−	−	−	+	−	+	−	−	−	−	−	− (0)
Cellobiose	−	−	−	−	−	+	−	−	−	−	−	−	−	− (0)
Arabinose	−	−	−	+	+	−	+	−	−	−	+	+	+	− (0)
Salicin	−	−	−	−	−	−	−	−	−	−	−	−	−	− (0)
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	− (0)
Deoxyribose	−	−	−	−	−	−	−	−	−	−	−	−	−	− (0)
Glucose, gas	−	−	−	−	−	−	−	−	−	−	−	−	−	− (0)
Glucose, acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+ (29)

[a] Symbols: +, positive result (with respect to acid production from carbohydrates, + indicates a final pH of <6.5); −, negative result.

[b] Numbers in parentheses are numbers of strains positive for the characters indicated.

[c] These strains required CO_2 on primary isolation.

[d] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

[e] PNPG, *p*-nitrophenyl-β-D-glucoside.

[f] Ability to produce infectious coryza in chickens.

solution to give a final concentration of 50 μg of NAD per ml of medium. Each of the tubes was inoculated with 0.05 ml (>10^6 colony-forming units per tube) of a bacterial suspension in 0.15 M NaCl made from 16- to 24-h-old BHIA-(V, S) cultures. Uninoculated tubes incubated under the same condition as described above served as controls. The final reactions were measured with a pH meter after 24 and 48 h of incubation.

The pathogenicity tests were performed as described by Hinz (11). Eight-week-old chickens free of avian encephalomyelitis virus, adenovirus, infectious bronchitis virus, Newcastle disease virus, respiratory enteric orphan virus, Marek disease herpesvirus, laryngotracheitis virus, Rous sarcoma virus, infectious bursal disease virus, influenza virus A, fowlpox virus, *Mycoplasma gallisepticum, Mycoplasma synoviae, Salmonella gallinarum, and S. pullorum* were used. *Haemophilus* cells used for inoculation of the chickens were obtained from 8- to 10-h-old BHIA-(V, S) cultures and were suspended in phosphate-buffered saline (pH 7.2) with 1% chicken serum and 10 μg of NAD per ml. *Haemophilus* cells used for inoculation were not washed. To test for the ability to cause infectious coryza, more than 5×10^7 colony-forming units in 0.05 ml of inoculum was instilled into nostrils and on the mucous membranes of the birds. Each of the *Haemophilus* strains was tested in five birds. A suspension of each *Haemophilus* strain inactivated at 100°C for 10 min was inoculated into two control chickens housed separately from the infected groups. Inoculated chickens were examined for clinical signs every day for 14 days. Necropsied birds were then examined for gross pathological changes, and samples from the respiratory tract were cultured for haemophili.

RESULTS

The characteristics of the *Haemophilus* strains studied are presented in Tables 2 and 3. On the basis of their characteristics, the strains studied fell into two groups: one group contains all of the strains of *H. paragallinarum*; the other contains the strains TS1 to TS12 inclusive and ATCC 14385 (TS13) which could not be assigned to one of the known *Haemophilus* species.

The characteristics of these unidentified strains (TS1 to TS13, inclusive) are as follows: Gram-negative, coccoid to pleomorphic, nonmotile, nonsporeforming rods (0.4 to 0.5 by 0.9 to 3.0 μm), which occur singly, in pairs, and in filamentous forms. Strains TS1 to TS6, inclusive, and TS9 to TS12, inclusive, formed smooth, convex, grayish-white or yellowish opaque colonies with entire edges on BHIA-(V, S). Colonies of strains TS7 and TS8 were wrinkled and gave lumpy suspensions in a 0.15 M NaCl solution. TS13 formed rough colonies with a granular surface. All of the strains except TS13 were encapsulated and showed iridescence on transparent solid media in oblique transmitted light

TABLE 3. *Characteristics useful in differentiating Haemophilus avium sp. nov. from H. paragallinarum*[a]

Characteristic	*H. avium*	*H. paragallinarum*
Aerobic growth	+/−	−
CO_2 requirement	−/+	+
Serum improves growth	−	+
Yellow-pigment production	+/−	−
Catalase	+	−
Alkaline phosphatase	+	−
Acid from:		
Trehalose	+	−
Galactose	+	−
α-Glucosidase (PNPG)	+	−/+
Pathogenicity[b]	−	+

[a] Symbols: +/−, most strains are positive; −/+, most strains are negative; +, positive result; −, negative result.

[b] Produces infectious coryza in chickens.

throughout the first 8 to 14 h of incubation. Iridescence disappeared completely after 36 h of incubation. Most of the strains did not require CO_2 for luxuriant growth in serial passages on solid media. However, strains TS10 and TS11 required increased CO_2 tension on primary isolation. Subcultures made from single colonies of those isolates on BHIA-(V, S) gave aerobic growth of a few colonies which did not require increased CO_2 tension in continued passages. It is probable that this adaptation was caused by dissociation. Since the properties of TS10 and TS11 were otherwise similar to those of the other strains, they were retained in the group of unidentified strains. All of the strains showed satellite phenomenon after microaerobic or aerobic incubation on ox blood agar streaked with a strain of *Staphylococcus aureus* as a V-factor feeder. They required the V factor but not the X factor or serum for growth. X-factor independence was confirmed by the ability of these strains to carry out biosynthesis of porphyrin from δ-aminolevulinic acid. In phenol red base, acid was produced from carbohydrates in an amount sufficient to give a clear-cut change of the indicator (pH < 6.5) after 1 to 2 days of incubation. The decrease of pH in carbohydrate- and serum-free phenol red broth produced by some of the unidentified strains reached only 0.6 U. All strains were catalase and phosphatase positive and produced acid from trehalose and galactose; some of the strains also produced acid from lactose, cellobiose, and arabinose. Four of the 13 strains produced β-galactosidase. In tryptose peptone broth, used as the basal medium for sugar fermentation in a previous study by Hinz (9), fewer strains produced acid from mal-

tose, dextrin, and xylose than in phenol red basal medium. None of the strains produced clinical or pathological signs in chickens. Infectious coryza could not be produced with any of these strains.

DISCUSSION

At present there is general agreement that the genus *Haemophilus* should be restricted to gram-negative, nonmotile, nonsporeforming rods with a requirement for hemin or other porphyrins (X factor) and/or for NAD (V factor) or other definable coenzyme-like substances (3, 29, 30). Results obtained in this study indicate the existence of a new species of the genus *Haemophilus* for which the name *Haemophilus avium* (a'vi.um. L.n. *avis* a bird; L. gen. pl. n. *avium* of birds) is proposed. This species differs from *H. gallinarum* and *H. paragallinarum* in its physiological, biochemical, and serological features and by its inability to cause infectious coryza of chickens (4, 9–12, 21, 23, 24, 28, 30). The characteristics which distinguish *H. avium* from *H. paragallinarum* are presented in Table 3. Some properties of *H. avium* are identical with those of *H. parasuis* and *H. parainfluenzae* but not with those of other known *Haemophilus* species. However, the data presented by Kilian (16) show that *H. parasuis* differs from *H. avium* in its α-fucosidase activity and by its weak or lack of fermentation of carbohydrates and from *H. parainfluenzae* in its oxidase and urease activities and negative α-glucosidase reaction. ATCC 14385, previously identified as a strain of *H. gallinarum,* is here identified as a member of *H. avium;* it has the same physiological and biochemical properties as described for it by Kilian (16) under the strain number HK 381 (= NCTC 3438 = ATCC 14385).

IPDH 2654, here designated as the type strain of *H. avium,* has been deposited in the American Type Culture Collection (ATCC) under the number 29546.

REPRINT REQUESTS

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