Avibacterium endocarditidis sp. nov., isolated from valvular endocarditis in chickens

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A novel species of the *Pasteurellaceae*, *Avibacterium endocarditidis* sp. nov., is proposed based upon characterization of 27 isolates from valvular endocarditis in adult broiler parents. All isolates shared the same PFGE type after digestion of DNA with *Sma*I and *Xba*I. In addition, all isolates meet the phenotypic characters for the genus *Avibacterium*. Separation of the novel species from other species of *Avibacterium* was possible by means of tests for catalase, symbiotic growth, aerobic growth on agar, acid production from glycerol, xylitol, (+)-L-arabinose, (–)-D-mannitol, (–)-D-sorbitol, (–)-L-fucose, (+)-D-galactose, maltose, trehalose, raffinose and dextrin in addition to reactions with ONPG (β -galactosidase) and PNPG (α -glucosidase). The closest relationship was observed with *Avibacterium gallinarum* which, however, can be separated from *Avibacterium endocarditidis* in acid production from (–)-D-mannitol, (–)-D-sorbitol and (–)-L-fucose. The highest 16S rRNA gene sequence similarity (98.4%) was found to strain Modesto, belonging to serogroup C of *Avibacterium paragallinarum*. *recN* gene DNA sequence similarities corrected by the formula of Zeigler (2003) (*Int J Syst Evol Microbiol* **53**, 1893–1900) documented 85% or less DNA sequence similarity between the type strain of *Avibacterium endocarditidis* and species of *Avibacterium*, confirming the separate species status of this taxon according to the multilocus sequence analysis method of Kuhnert & Korczak (2006) (*Microbiology* **152**, 2537–2548). The type strain of *Avibacterium endocarditidis* sp. nov., strain 20186H4H1^T (=CCUG 52860^T =DSM 18224^T), was isolated from valvular endocarditis of a chicken in Denmark in 2004.

Within the *Pasteurellaceae*, two new genera *Gallibacterium* (Christensen *et al.*, 2003a) and *Volucribacter* (Christensen *et al.*, 2004) have recently been established within the avian 16S rRNA cluster 18 of Olsen *et al.* (2005). Four named species as well as an unnamed taxon were subsequently transferred to a new genus *Avibacterium* as *Avibacterium gallinarum* gen. nov., comb. nov., *Avibacterium paragallinarum* comb. nov., *Avibacterium avium* comb. nov., *Avibacterium volantium* comb. nov. and *Avibacterium* species A (Blackall *et al.*, 2005). While all of the named species of *Avibacterium* are routinely encountered in the investigation of upper respiratory tract diseases in birds, only *Avibacterium paragallinarum* is regarded as a primary pathogen, being the causative agent of infectious coryza

of chickens (Blackall, 1999). *Avibacterium avium*, *Avibacterium volantium* and *Avibacterium* sp. A are normally regarded as part of the commensal microbiota of the upper respiratory tract of chickens and other birds, but are occasionally implicated in various pathologies (Hinz, 1980; Hinz & Kunjara, 1977; Mutters *et al.*, 1985; Bisgaard, 1993). The only species of *Avibacterium* involved in systemic infections in addition to upper respiratory tract infections seems to be *Avibacterium gallinarum*, affecting gallinaceous birds (Christensen *et al.*, 2002a; Bisgaard *et al.*, 2005). In one report, *Avibacterium paragallinarum* has been isolated from lung, kidney and liver, probably related to systemic infection (Sandoval *et al.*, 1994). A novel non-V-factor-requiring group of the *Pasteurellaceae* showing phenotypic characters similar to those of *Avibacterium* and tentatively named taxon 50 was recently isolated from valvular endocarditis and septicaemia in broiler parents (M. Bisgaard, unpublished data). Based on phenotypic and genotypic data including PFGE and sequencing of 16S rRNA and *recN* genes, we propose to accommodate taxon 50 within the genus *Avibacterium* as a separate species.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 20186H4H1^T is DQ465412, and those for the *recN* sequences of strain 20186H4H1^T, *A. volantium* NCTC 3438^T, *A. paragallinarum* NCTC 11296^T and *Avibacterium* sp. A strain CCUG 18782 are DQ899746–DQ899749, respectively.

PFGE results and a *recN* sequence-based maximum-likelihood tree are available as supplementary material with the online version of this paper.

Bacterial strains and growth conditions

A total of 27 isolates from 22 adult broiler parents of the same flock were sampled over a period of 10 weeks and characterized. Twenty isolates were obtained from valvular endocarditis. Eighteen were recorded during production, while two cases were recorded among dead hens left in the house after collection of birds for slaughtering. Two birds with endocarditis allowed two isolates from the liver and two isolates from the spleen to be investigated. Finally, two isolates were obtained from degenerative arthritis and a single isolate originated from chronic adhesive pericarditis of birds without valvular endocarditis (M. Bisgaard, unpublished data). All isolates were grown aerobically on 5 % bovine blood agar (blood agar base, CM 55; Oxoid) at 37 °C unless otherwise stated.

Phenotypic characterization

Classical phenotypic tests were performed as reported previously (Bisgaard *et al.*, 1991). Additional characterization included API 20NE tests, which were carried out according to the recommendations of the manufacturer (bioMérieux).

All 27 isolates tentatively classified as taxon 50 were Gram-negative, non-motile, pleomorphic rods that were catalase- and oxidase-positive and produced acid from glucose without gas in Hugh & Leifson's medium. Haemolysis of bovine red-blood cells was negative and symbiotic growth was not observed. Negative reactions were also observed in Simmons' citrate, mucate, malonate, methyl red and Voges-Proskauer tests, production of H₂S in triple sugar iron (TSI) medium, KCN growth, urease, arginine dihydrolase, lysine and ornithine decarboxylases, phenylalanine deaminase, indole, gelatinase and hydrolysis of Tweens 20 and 80. Growth was not observed on MacConkey agar. All isolates were alanine aminopeptidase-positive and reduced nitrate without gas production. Aerobic growth without pigment formation was observed on agar. The porphyrin and phosphatase tests were positive. All isolates produced acid from glycerol, (–)-D-ribose, (–)-D-mannitol, (–)-D-sorbitol, (–)-D-fructose, (+)-D-galactose, (+)-D-glucose, (+)-D-mannose, maltose, sucrose, trehalose, raffinose and dextrin. Late production of acid from (+)-D-xylose and inositol might be observed, since these reactions were inconsistent with the strains tested. No acid was produced by any isolate from *meso*-erythritol, adonitol, (+)-D-arabitol, xylitol, (+)-L-arabinose, (–)-D-arabinose, (–)-L-xylose, dulcitol, (+)-D-fucose, (–)-L-fucose, (+)-L-rhamnose, (–)-L-sorbose, cellobiose, lactose, (+)-D-melibiose, (+)-D-melezitose, (+)-D-glycogen, inulin, aesculin, amygdalin, arbutin, gentiobiose, salicin or (+)-D-turanose. All isolates were ONPG- (β -galactosidase) and PNPG- (α -glucosidase) positive and NPG- (β -glucosidase), ONPF- (α -fucosidase), ONPX- (β -xylosidase), PGUA- (β -glucuronidase), α -galactosidase- and α -mannosidase-negative.

In addition to confirming reactions for oxidase, indole, arginine dihydrolase, urease, hydrolysis of gelatin,

β -glucosidase and β -galactosidase, API 20NE showed that all isolates of taxon 50 did not reduce nitrates to nitrogen and were unable to assimilate arabinose, caprate, adipate, citrate or phenylacetate, while glucose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate and malate were assimilated.

Key characters that separate taxon 50 from other taxa within the genus *Avibacterium* are given in Table 1. From three to nine phenotypic characters separate taxon 50 from previously reported taxa of *Avibacterium*. The closest relationship was observed for *Avibacterium gallinarum* which, however, can be separated from taxon 50 by acid production from (–)-D-mannitol, (–)-D-sorbitol and (–)-L-fucose. Phenotypic characters that separate the genus *Avibacterium* from other genera of the *Pasteurellaceae* were recently published by Blackall *et al.* (2005).

Pulsed field gel electrophoresis (PFGE)

PFGE using *Sma*I and *Xba*I for digestion of DNA was performed as reported previously (Ojeniyi *et al.*, 1991; Christensen *et al.*, 2003a; Chadfield *et al.*, 2007). The size marker N0350S (New England Biolabs) was used for comparison. All isolates of taxon 50 shared the same PFGE type (Supplementary Fig. S1 available in IJSEM Online), indicative of a clonal outbreak of valvular endocarditis. However, since the isolates reported represent the first and only isolates described, additional isolates are needed to allow comments on the genetic diversity of this novel taxon of the *Pasteurellaceae*. The type strain of *Avibacterium gallinarum* showed a divergent *Xba*I profile, sharing only two bands with taxon 50 (Supplementary Fig. S1).

All 27 isolates were isolated from the same flock over a period of 10 weeks and shared a common phenotype and genotype. To describe a novel species, greater diversity would have been desirable (Christensen *et al.*, 2007). However, the minimal standards for description of novel taxa of the *Pasteurellaceae* recommend naming of species of particular pathogenic importance irrespective of whether they cover geographical and ecological diversity (Christensen *et al.*, 2007). By doing so, type strains are also made available to the public for further characterization, including investigations of mechanisms of pathogenicity.

16S rRNA gene sequencing

16S rRNA gene sequencing of strain 20186H4H1^T was performed as reported previously (Angen *et al.*, 2003; Christensen *et al.*, 2002b). BLAST searching (Altschul *et al.*, 1997) in GenBank (Benson *et al.*, 2006) showed that no other sequences had been deposited with high similarity to strain 20186H4H1^T. Pairwise comparisons for similarity were performed by the program WATER included in EMBOSS (Rice *et al.*, 2000). The 16S rRNA gene sequence for strain 20186H4H1^T was obtained in the region covering *Escherichia coli* positions 194–1412.

Table 1. Key characters for separation of taxa within the genus *Avibacterium* Blackall *et al.* 2005

Taxa: 1, *Avibacterium gallinarum* (data from Bisgaard *et al.*, 2005; Christensen *et al.*, 2002a); 2, *Avibacterium paragallinarum* biovar 1 (Blackall & Reid, 1982; Hinz, 1980); 3, *Avibacterium paragallinarum* biovar 2 (Blackall *et al.*, 2005); 4, *Avibacterium avium* (Mutters *et al.*, 1985); 5, *Avibacterium volantium* (Mutters *et al.*, 1985); 6, *Avibacterium endocarditidis* sp. nov. (Bisgaard taxon 50) (data from this study); 7, *Avibacterium* sp. A (Mutters *et al.*, 1985). Characters are scored as: +, 90 % or more of strains positive within 1–2 days; (+), 90 % or more strains positive within 3–14 days; d, 11–89 % of strains positive within 14 days; w, weakly positive within 14 days; –, less than 10 % of strains positive within 14 days; ND, no data.

Test	1	2	3	4	5	6	7
Catalase	+	–	–	+	+	+	+
Symbiotic growth	–	+	–	+	+	–	+
Aerobic growth on agar	+	–	+	+	+	+	+
Pigment, yellow	–	–	–	d	d	–	d
Acid from:							
Glycerol	d	–	–	ND	–	(+)	–
Xylitol	–	–	–	ND	–	–	(+)
(+)–L–Arabinose	–	–	–	–	–	–	+
(–)–D–Mannitol	–	+/(+)	+/(+)	–	+	+	d
(–)–D–Sorbitol	–	d	+/(+)	–	d	+	–
(–)–L–Fucose	w/(+)	–	w/(+)	ND	(+)	–	–
(+)–D–Galactose	+	–	–	+	+	(+)	+
ONPG (β -galactosidase)	d	–	–	–	+	+	d
Maltose	+	(+)	–	–	+	+	d
Trehalose	+	–	–	+	+	+	+
Raffinose	w/(+)	–	–	ND	–	(+)	(+)
Dextrin	+	w/(+)	–	+	+	+	–
PNPG (α -glucosidase)	+	–	–	+	+	+	+

The highest 16S rRNA gene sequence similarity between this strain and other taxa of the *Pasteurellaceae* was obtained to species of *Avibacterium*. The highest similarity (98.4 %) was obtained to strain Modesto, belonging to serogroup C of *Avibacterium paragallinarum* (GenBank accession no. AY498870). Similarities to other members of *Avibacterium* ranged between 96.5 %, to the type strain of *Avibacterium gallinarum* (GenBank accession no. M75059), and 97.9 %, to strain SA7177 of biovar 2 of *Avibacterium paragallinarum* (accession no. AY498871) as well as strain CCUG 18782 of *Avibacterium* sp. A (accession no. M75055). Similarities to strains Wuthe DS 1801/91 (GenBank accession no. AF224311) and CCUG 19808 (accession no. AF224312), closely related to *Avibacterium*, were 93.6 and 92.1 %, respectively. Outside *Avibacterium*, similarities to the type strains of *Volucribacter psittacida* and *Volucribacter amazonae* were 94 and 93.5 %, respectively, and the highest similarity to the taxon 2 and 3 complex of Bisgaard was 93.8 %, as observed to the reference strain NCTC 11412 of taxon 3 (GenBank accession no. L06079), while 93.1 % similarity was observed to the type strain of *Gallibacterium anatis* (accession no. AF228001). The highest similarity outside the avian 16S rRNA cluster (Olsen *et al.*, 2005) was obtained to the type strain of [*Pasteurella*] *caballi*, at 95.4 % (GenBank accession no.

AF224291) [brackets around genus names indicate species suggested to be excluded from the respective genera in accordance with the terminology of Mutters *et al.* (1989)].

A multiple alignment was constructed by CLUSTAL_X (Thompson *et al.*, 1997) and included the region between *E. coli* positions 194 and 1391 of the *rrnB* gene, with 1175 positions left after removal of gapped columns and 196 data patterns analysed. Maximum-likelihood analysis including bootstrap analysis was performed by fastDNAm1 (Olsen *et al.*, 1994; Felsenstein, 1995) run on a Linux 7.2-compatible server. The analysis was run with a transition/transversion ratio of 1.5.

The phylogenetic analysis shown in Fig. 1 was obtained with 22 sequences selected to include all *Avibacterium* type strains within the avian 16S rRNA cluster of the *Pasteurellaceae* (Olsen *et al.*, 2005) and type species of other genera within the *Pasteurellaceae*. The novel species was found to belong to the monophyletic group including taxa of *Avibacterium*, confirming the 16S rRNA gene sequence similarities. Monophyly for *Avibacterium* was confirmed in accordance with Christensen *et al.* (2003b) and Blackall *et al.* (2005). The low bootstrap value of the group of only 57 % and the lack of documentation of the avian 16S rRNA group (Olsen *et al.*, 2005) was probably related to an

Fig. 1. Phylogenetic relationships between taxon 50 (*Avibacterium endocarditidis* sp. nov.) and representative members of the *Pasteurellaceae* with a focus on *Avibacterium* and related taxa and avian cluster 18 (Olsen *et al.*, 2005) based on maximum-likelihood analysis of 16S rRNA gene sequences. Support for monophyletic groups by bootstrap analysis is indicated as numbers out of 100. The strain in bold has been sequenced in the present investigation. Bar, evolutionary distance of 0.01 (represents sequence variation considering the models for nucleotide substitution and tree-shape used in the maximum-likelihood analysis).

unbalanced representation of members of the *Pasteurellaceae* with a focus on only the type strains of type species of genera.

recN sequencing and analysis

16S rRNA gene sequence analysis is insufficient to separate taxa within *Avibacterium* (Blackall *et al.*, 2005) and, since the species status of taxon 50 could not be documented unequivocally through 16S rRNA gene sequence comparison, comparison of the *recN* sequence of 20186H4H1^T to those of the type strains of the species of *Avibacterium* and strain CCUG 18782 of *Avibacterium* sp. A was performed. Sequencing of strain 20186H4H1^T (*Avibacterium endocarditidis* sp. nov.), *Avibacterium paragallinarum* NCTC 11296^T, *Avibacterium volantium* NCTC 3438^T and *Avibacterium* sp. A strain CCUG 18782 was carried out as reported by Kuhnert & Korczak (2006). In addition to the primers listed by Kuhnert & Korczak (2006), the sequencing primers 5'-AAAATGCGCAAGCTTTGAGT and 5'-ACACGGCGTAAATACAG were used to determine the 3' and 5' regions, respectively, of the *recN* sequence. DNA sequences were obtained of the region 94–1255 of the *recN* gene (numbered according to annotation of the genomic sequence of *Pasteurella multocida* strain Pm70 deposited under GenBank accession no. AE004439). The type strain of *Avibacterium avium* did not generate a PCR product with the *recN* primers stated above, indicating significant differences from the other species of *Avibacterium*, and this species was not included in the analysis. The published sequences of the type strains of *Avibacterium gallinarum* and *Pasteurella multocida* and of *Volucrobacter psittacida* strain 101 were also included in the comparison. The phylogenetic analysis was performed as reported for 16S rRNA gene sequences, including 1255 positions and the use

of a transition/transversion ratio of 1.1, which was found to maximize the likelihood. The phylogenetic tree shown in Supplementary Fig. S2 shows the unique position of taxon 50 within *Avibacterium*.

Similarities of 41, 84 and 85 % were calculated using the formula for conversion given by Zeigler, (2003) between strain 20186H4H1^T and the type strains of *Avibacterium paragallinarum*, *Avibacterium gallinarum* and *Avibacterium volantium*, respectively, whereas the similarity to *Avibacterium* sp. A was 83 %. DNA sequence similarity values calculated from *recN* sequences of 17 strains of *Actinobacillus capsulatus* representing seven different countries and four continents showed similarities between 94 and 95 % (Kuhnert *et al.*, 2007), while similarity values between different serovars of *Actinobacillus pleuropneumoniae* varied between 91 and 95 % (Kuhnert & Korczak, 2006). Comparison of *recN* in the present study therefore clearly confirmed the species status of taxon 50 (*Avibacterium endocarditidis* sp. nov.).

Description of *Avibacterium endocarditidis* sp. nov.

Avibacterium endocarditidis (en.do.car.di'ti.dis. N.L. n. *endocarditidis* endocarditis, the medical term for an infection of the inner heart; N.L. gen. n. *endocarditidis* of endocarditis).

Growth on blood agar is non-symbiotic, isolates forming regular, circular, slightly raised, non-haemolytic colonies with an entire margin. The surface of the colonies is smooth, shiny and opaque with a greyish tinge. Colonies have a diameter of 1.0–1.5 mm after aerobic incubation for 24 h at 37 °C. The consistency of the colonies is unguent-like and colonies do not adhere to the agar surface. All

isolates meet the phenotypic characters for the genus *Avibacterium* reported previously (Blackall *et al.*, 2005). Catalase is produced. Acid is produced from glycerol, (–)-D-mannitol, (–)-D-sorbitol, (+)-D-galactose, maltose, trehalose, raffinose and dextrin but not from xylitol, (+)-L-arabinose or (–)-L-fucose. ONPG and PNP tests are positive. The characters stated allow separation from other species of *Avibacterium*. Detailed phenotypic characters for all isolates including the type strain are given under phenotypic characterization.

The type strain, 20186H4H1^T (=CCUG 52860^T =DSM 18224^T), was isolated from valvular endocarditis of a chicken in Denmark in 2004.

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