Campylobacter avium sp. nov., a hippurate-positive species isolated from poultry

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Three strains of an unusual hippurate-positive *Campylobacter* species were isolated at 37 °C from caecal contents of broiler chickens and a turkey. All strains were initially identified as *Campylobacter* by means of genus-specific PCR, but none was further identified using specific PCRs for known thermophilic species. Phylogenetic analyses based on 16S rRNA, *rpoB* and *groEL* gene sequences revealed that these strains formed a robust clade distinct from other *Campylobacter* species. Amplified fragment length polymorphism analysis and whole-cell protein electrophoresis were subsequently carried out and confirmed the divergence between the avian strains and other taxa. These data indicate that the unidentified *Campylobacter* strains belong to a novel taxon which could be distinguished from other campylobacters through its phenotypic and genotypic characteristics. The name *Campylobacter avium* sp. nov., is proposed for the novel species, with the type strain 86/06^T (=LMG 24591^T =CCUG 56292^T).

The genus *Campylobacter* was proposed by Sebald & Véron (1963). The genus has since been expanded, with species originating from mammals and birds, and now includes 19 species and 6 subspecies (Foster *et al.*, 2004; Vandamme *et al.*, 2005; Inglis *et al.*, 2007; Zanoni *et al.*, 2009). In the present study, we report a polyphasic taxonomic characterization of three unusual hippurate hydrolase-producing *Campylobacter* strains that were recovered from the caecal contents of poultry.

In the course of bacteriological investigations intended to define the prevalence of *Helicobacter pullorum* in poultry, seven unusual hippurate-positive *Campylobacter* isolates were recovered from caecal contents of six broiler chickens and one turkey originating from three different farms in Italy. Caeca were collected at the slaughterhouse between July and October 2006. Campylobacters were isolated after 3–4 days of incubation at 37 °C in a microaerobic atmosphere with hydrogen on Brucella sheep blood agar [Brucella broth (BBL) with 1.5 % Bacto agar (Difco) and 5 % sheep blood] using a filter method (Zanoni *et al.* 2007). The microaerobic atmosphere with hydrogen was obtained by the gas replacement method using an anaerobic gas mixture (10 % H_2, 10 % CO_2, 80 % N_2) as described by Bolton *et al.* (1992). After 3–4 days of incubation on Brucella sheep blood agar, growth appeared as a spreading layer on the agar medium. Single colonies were not seen. Pure cultures were obtained after dilution and repeated subculturing. Following subculturing after 48 h of incubation at 37 °C, colonies appeared flat, greyish and finely granular with an irregular edge, and showed a tendency to spread along the direction of the streak and to swarm and coalesce. Cells were Gram-negative, sigmoid to allantoid in shape, 1–3 μm long and 0.2–0.4 μm wide, when observed after Gram staining, and appeared coccoid after 4–5 days of incubation.

For genotyping analysis, bacterial DNA was extracted by using a ChargeSwitch gDNA Mini Bacteria kit (Invitrogen Life Technologies).

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Abbreviation: AFLP, amplified fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB* and *groEL* gene sequences of strains 86/06^T, 24/06 and 87/06 are respectively EU623473–EU623475 (16S rRNA gene), EU643476, EU643478 and EU643477 (*rpoB*) and EU636014, EU636013 and EU636812 (*groEL*).

Neighbour-joining dendrograms based on partial *rpoB* and *groEL* sequences and a UPGMA dendrogram based on analysis of protein profiles of strains of *Campylobacter avium* sp. nov. and other *Campylobacter* species are available as supplementary material with the online version of this paper.

Isolates were initially identified as *Campylobacter jejuni* because of their ability to hydrolyse hippurate. To confirm the identification, isolates were further analysed by means of a genus-specific PCR for the genus *Campylobacter* (Linton *et al.*, 1996) and several species-specific PCRs for different *Campylobacter* species (Denis *et al.*, 1999; Lawson *et al.*, 1997; Linton *et al.*, 1996). They were confirmed as *Campylobacter*, but could not be identified at the species level. The diversity among the unidentified *Campylobacter* isolates was assessed by visual analysis of whole-cell protein profiles in one-dimensional SDS-PAGE, carried out as described by Zanoni *et al.* (2007). All seven *Campylobacter* isolates showed almost identical protein profiles (data not shown), suggesting that they belong to the same taxon.

To obtain a more detailed identification, we selected three isolates (86/06^T, 87/06 and 24/06) from different hosts (broiler chicken and turkey) and from different regions in Italy, thereby representing a geographically and epidemiologically independent set of isolates.

In order to define the taxonomic position of the avian isolates, a phylogenetic analysis based on 16S rRNA gene sequences was carried out. The nearly complete 16S rRNA gene was amplified using universal primers p27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and p1492r (5′-TACGGCTACCTTGTTACGACT-5′) and the PCR-amplified template was sequenced by primer walking (Primm s.r.l.). Sequences were assembled with Vecton NTI software (Invitrogen) and then aligned in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) by CLUSTAL W using *Campylobacter* reference sequences obtained from GenBank. The alignment was adjusted visually removing intervening sequence regions and unknown bases. Finally, data were corrected for multiple base changes by the method of Jukes & Cantor (1969). A phylogenetic tree was constructed in MEGA3 (http://www.megasoftware.net/) using the neighbour-joining method. Bootstrap analysis was performed with 1000 resampled datasets.

A fragment of approximately 1300 bp of the 16S rRNA gene was obtained. Analysis of the 16S rRNA gene sequences using MEGABLAST (http://www.ncbi.nlm.nih.gov/BLAST/) indicated that the isolates were most closely related to taxa within the genus *Campylobacter*, confirming their generic identification. Pairwise comparisons of 16S rRNA gene sequences showed that the three isolates were genetically highly related, exhibiting 99.1–99.9 % sequence similarity. Furthermore, the neighbour-joining dendrogram (Fig. 1) indicated that they formed a robust clade (100 % bootstrap support) which was clearly distinct from other *Campylobacter* species. Pairwise sequence comparisons of 86/06^T with type strains of the most closely related species revealed similarities of 96.6, 95.5, 94.9 and 94.2 % with *Campylobacter upsaliensis*, *C. helveticus*, *C. cuniculorum* and *C. jejuni*, respectively.

The phylogenetic relationships of these bacteria were further examined by *rpoB* (Korczak *et al.* 2006) and *groEL* (Kärenlampi *et al.* 2004) sequence analysis. Sequences were processed as described above. Phylogenetic trees based on partial *rpoB* and *groEL* nucleotide sequences are shown in Supplementary Figs S1 and S2 (available in IJSEM Online). In both trees, the unidentified strains clustered together in a tight clade supported by a high bootstrap value (100 %) and clearly separated from all *Campylobacter* species. The *rpoB* sequences of the avian strains were 99–100 % similar, while similarity values towards those of other *Campylobacter* species varied from 64 to 74.8 %. Likewise, pairwise comparisons of the *groEL* sequences among the avian strains yielded similarity of 99.2–100 %, while values towards other *Campylobacter* species were less than 82.4 %. Like Korczak *et al.* (2006) and Kärenlampi *et al.* (2004), we observed a good congruence

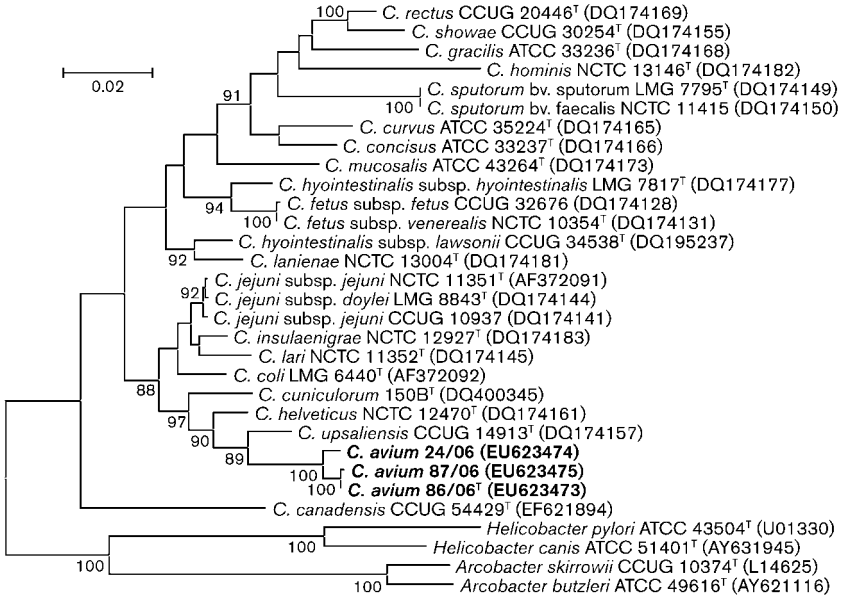

Fig. 1. Unrooted tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of the three strains of *C. avium* sp. nov. Bar, 0.02 nucleotide substitutions per base. Numbers at nodes (⩾88 %) indicate support for the internal branches within the tree obtained by bootstrap analysis (percentages of 1000 bootstraps).

between *rpoB*, *groEL* and 16S rRNA gene sequence results, since each of the phylogenetic trees showed a similar topology. However, compared to the 16S rRNA gene, *rpoB* and *groEL* sequence analyses showed higher resolution as a result of their lower interspecies similarity.

Although all the sequence data demonstrated that the three isolates represented a coherent taxon, amplified fragment length polymorphism (AFLP) analysis and whole-cell protein electrophoresis were performed to further examine the relationships between the strains.

AFLP analysis was performed as described by Debruyne *et al.* (2009). In brief, 1 μg genomic DNA was digested with the *Hin*dIII–*Hha*I restriction enzyme combination. After digestion, site-specific adaptors were ligated to the restriction fragments and primers complementary to the adaptor and restriction site sequence were used in subsequent preselective and selective PCRs. The amplified and fluorescently labelled fragments were loaded on a denaturing polyacrylamide gel on an ABI Prism 377 automated sequencer. GENESCAN version 3.1 (Applied Biosystems) was used for data collection, and the generated profiles were imported, using the CrvConv filter, in BioNumerics version 4.61 (Applied Maths) for normalization and further analysis. Similarity between normalized profiles was determined by Pearson's product–moment correlation coefficient and a UPGMA dendrogram was constructed. Numerical analysis of the AFLP profiles obtained (Fig. 2) differentiated the avian taxon from other *Campylobacter* species. Moreover, the AFLP profiles of the three strains were all different, thereby demonstrating that the isolates represent different clones.

Whole-cell protein profile analysis was performed using SDS-PAGE as described by Pot *et al.* (1994). For this analysis, strains were grown microaerobically on Mueller–Hinton agar (Oxoid) supplement with 5 % (v/v) defibrinated horse blood and incubated microaerobically at 37 °C for 48 h. Whole-cell protein profiles of *Campylobacter* reference strains were available from previous studies (Vandamme *et al.*, 1991). Densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis, were performed using the GelCompar software package (version 4.2; Applied Maths). For whole-cell protein SDS-PAGE analysis, similarity of the obtained normalized SDS-PAGE patterns was determined by Pearson's product–moment correlation coefficient, after which clustering was performed by UPGMA. The dendrogram obtained by numerical analysis of the protein profiles of the three avian strains and of *Campylobacter* reference strains is shown in Supplementary Fig. S3. The three strains grouped in a single cluster above a similarity level of 94 % and were clearly distinct from the other *Campylobacter* species.

For the determination of G+C content, DNA was enzymically degraded into nucleosides as described by Mesbah & Whitman (1989). The nucleoside mixture was separated by HPLC using a Waters SymmetryShield C8 column maintained at 37 °C. The solvent was 0.02 M $(NH_4)H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated λ phage DNA (Sigma) was used as the calibration reference. The G+C content of the DNA of strain 86/06^T was 35 mol%. This value is within the range of 28–47 mol% reported for genus *Campylobacter* (Vandamme *et al.*, 2005).

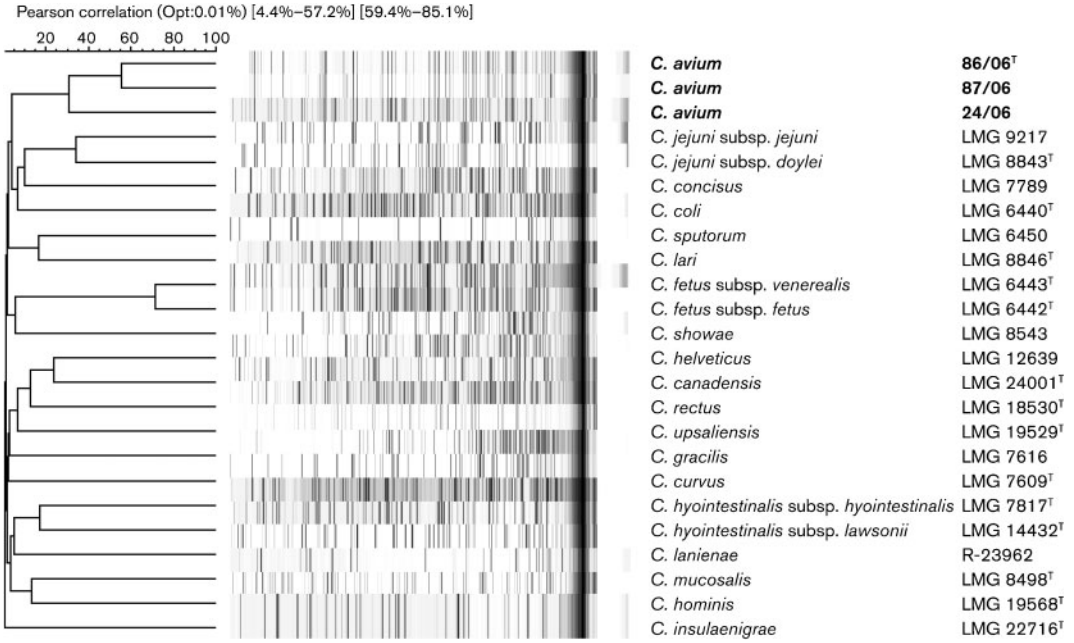

Fig. 2. Dendrogram of the three strains of *C. avium* sp. nov. based on UPGMA cluster analysis of AFLP profiles.

Table 1. Phenotypic characteristics of *Campylobacter* species

Taxa: 1, *C. avium* sp. nov.; 2, *C. canadensis*; 3, *C. coli*; 4, *C. concisus*; 5, *C. cuniculorum*; 6, *C. curvus*; 7, *C. fetus* subsp. *fetus*; 8, *C. fetus* subsp. *venerealis*; 9, *C. gracilis*; 10, *C. helveticus*; 11, *C. hominis*; 12, *C. hyointestinalis* subsp. *hyointestinalis*; 13, *C. hyointestinalis* subsp. *lawsonii*; 14, *C. insulanigrae*; 15, *C. jejuni* subsp. *doylei*; 16, *C. jejuni* subsp. *jejuni*; 17, *C. lanienae*; 18, *C. lari*; 19, *C. mucosalis*; 20, *C. rectus*; 21, *C. showae*; 22, *C. sputorum*; 23, *C. upsaliensis*. Data for reference species were taken from On *et al.* (1996), Foster *et al.* (2004), Vandamme *et al.* (2005), Inglis *et al.* (2007) and Zanoni *et al.* (2009). All taxa are negative for aerobic growth at 37 °C. +, 90–100 % of strains positive; −, 0–10 % of strains positive; (+), 75–89 % strains positive; (−), 11–25 % of strains positive; V, 26–74 % of strains positive; W, weakly positive; NA, no data available. CCDA, Charcoal cefoperazone deoxycholate agar (Oxoid); TTC, triphenyl tetrazolium chloride.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
α-Haemolysis	−	−	(−)	(−)	+	(−)	−	V	−	+	NA	V	V	NA	+	+	+	V	−	+	+	+	+
Oxidase	+	+	+	V	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	V	+	+
Catalase	W	V	+	−	+	−	+	(+)	V	−	−	+	+	+	V	+	+	+	−	(−)	+	V	−
Alkaline phosphatase	−	−	−	V	−	V	−	−	−	−	−	−	(−)	NA	−	−	+	−	(+)	−	−	−	−
γ-Glutamyltranspeptidase	−	(+)	−	−	−	NA	−	NA	NA	−	NA	−	−	NA	−	−	NA	−	NA	NA	NA	−	−
Urease production	−	V	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	V	−	−	−	V*	−
Hippurate hydrolysis	+	−	−	−	−	(−)	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
Indoxyl acetate hydrolysis	+	−	+	−	+	V	−	−	V	+	−	−	−	−	+	+	−	−	−	+	−	−	+
Nitrate reduction	+	V	+	(−)	+	+	+	+	(+)	+	−	+	+	+	−	+	+	+	−	+	+	+	+
Selenite reduction	−	NA	V	(−)	−	−	(+)	−	−	−	−	+	+	NA	−	+	+	+	−	+	+	+	+
TTC reduction	−	NA	+	−	V	V	−	−	−	−	NA	−	−	NA	V	+	NA	+	−	−	−	−	V
Trace H_2S on TSI agar	−	V	−	−	−	(−)	−	−	−	−	−	+	+	−	−	−	−	−	+	−	V	+	−
Growth at/in/on:																							
25 °C (microaerobic)	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
37 °C (microaerobic)	+	+	+	+	+	V	+	+	−	+	+	+	+	+	+	+	+	+	+	−	V	+	+
42 °C (microaerobic)	+	+	+	(+)	(+)	V	(+)	−	V	+	(−)	+	+	−	−	+	+	+	+	(−)	V	+	+
37 °C (anaerobic)	−	+	−	+	−	+	(−)	V	+	−	+	−	+	−	−	−	+	−	+	+	+	+	−
Nutrient agar	−	−	+	(−)	+	+	+	+	+	(+)	NA	+	+	NA	+	+	NA	+	+	(−)	V	+	+
CCDA	−	+	+	(−)	(+)	(+)	+	+	V	+	NA	+	+	NA	+	+	NA	+	+	−	+	(+)	+
MacConkey agar	−	+	V	−	−	(+)	(+)	V	(+)	−	−	V	V	NA	−	−	+	−	(+)	−	+	V	−
1 % Glycine	−	V	+	(−)	−	+	+	−	+	V	+	+	V	+	(−)	+	−	+	V	+	V	+	+
2 % NaCl	−	NA	−	(−)	−	V	−	−	V	−	+	−	−	−	−	−	−	(+)	+	V	+	+	−
1 % Bile	V	NA	(+)	−	V	−	+	+	−	+	NA	+	(+)	NA	+	+	NA	+	+	−	−	V	+
Requirement for H_2	V	−	−	+	−	+	−	−	+	−	+	V	V	NA	−	−	−	−	+	+	+	−	−
Resistance to:																							
Nalidixic acid	−	V	−	(+)	V	+	+	V	V	−	V	+	+	+	−	−	+	V	(+)	(+)	−	(+)	−
Cephalothin	+	−	+	−	(+)	−	−	−	−	−	−	(−)	−	+	−	+	+	+	−	−	−	−	(−)

The results of physiological characterization of the three avian strains, determined using standard methods (On & Holmes, 1991a, b; 1992; Ursing *et al.*, 1994; On *et al.*, 1996), are presented in Table 1 and in the species description. These characteristics allowed differentiation of the poultry strains from established *Campylobacter* species. Among the hippurate-positive species, the poultry strains can be distinguished from *C. jejuni* subsp. *jejuni* by their inability to grow in the presence of 1 % (w/v) glycine and to reduce selenite or triphenyl tetrazolium chloride and from *C. jejuni* subsp. *doylei* by their ability to grow at 42 °C and to reduce nitrate.

In conclusion, the results of this polyphasic taxonomic study indicate that the three strains recovered from caecal contents of poultry represent a unique *Campylobacter* species for which we propose the name *Campylobacter avium* sp. nov. Sequence analysis of *rpoB* and *groEL* genes and also AFLP and whole-cell protein profile analysis and traditional biochemical analysis allow the novel species to be distinguished from established species.

Campylobacters are the most common bacterial cause of human enteric infections worldwide. Species identification of human isolates is usually carried out in routine clinical microbiology laboratories by means of phenotypic methods. The ability of *C. jejuni* to hydrolyse hippurate (*N*-benzoylglycine) to benzoic acid and glycine is commonly used to distinguish it from other *Campylobacter* species, and only hippurate-negative strains are usually tested by molecular methods (Nakari *et al.*, 2008; Wainø *et al.*, 2003). Nakari *et al.* (2008) recently standardized the hippurate test by determining cell suspension turbidity limits using *C. jejuni* and *Campylobacter coli* reference strains. All strains of *C. avium* sp. nov. were also able to hydrolyse hippurate when tested with the low suspension turbidity described for *C. jejuni* by Nakari *et al.* (2008), thus confirming the previous results. Hippurate hydrolase activity in *C. jejuni* is due to the presence of an enzyme encoded by the *hipO* gene (Hani & Chan, 1995), and different *hipO*-based species-specific PCR tests for *C. jejuni* have been described (Slater & Owen, 1997; Burnett *et al.*, 2002; Bang *et al.*, 2002). None of these PCRs amplified the hippurate hydrolase gene of *C. avium* sp. nov. (data not shown). These results suggest that hippurate-positive campylobacters may erroneously be considered as *C. jejuni* if insufficient biochemical characterization or subsequent molecular confirmation is performed.

Description of *Campylobacter avium* sp. nov.

Campylobacter avium (a′vi.um. L. gen. pl. n. *avium* of birds).

Cells are spiral, Gram-negative rods, motile, 0.2–0.4 μm wide and 1–3 μm long. On Brucella sheep blood agar at 37 °C after 48 h under microaerobic conditions, colonies appeared non-α-haemolytic, flat, greyish and finely granular with an irregular edge and show a tendency to spread along the direction of the streak and to swarm and coalesce. Strictly microaerobic. Able to grow at 37 and 42 °C, but not at 25 °C or under anaerobic and aerobic conditions. Most strains do not require hydrogen to grow. Oxidase and weak catalase activity are observed, but not urease, γ-glutamyltranspeptidase or alkaline phosphatase. Strains hydrolyse hippurate and indoxyl acetate and reduce nitrate but not selenite or triphenyl tetrazolium chloride. Strains do not produce H_2S in TSI agar. Most strains grow in the presence of 1 % (w/v) bile. No growth occurs on nutrient agar without blood, on MacConkey agar or in the presence of 1 % (w/v) glycine or 2 % (w/v) NaCl. Growth on charcoal cefoperazone deoxycholate agar (CCDA; Oxoid) appears after 4–5 days of incubation, and growth on this medium is slightly restricted. Strains are susceptible to nalidixic acid (30 μg) and resistant to cephalothin (30 μg) by disc diffusion tests. Pathogenicity is unknown.

The type strain is 86/06^T (=LMG 24591^T =CCUG 56292^T), which was isolated from a broiler chicken in Italy in 2006. Strains 24/06 (=CCUG 56294) and 87/06 (=LMG 24592 =CCUG 56293) are also strains of the species. Strains have been recovered from poultry caecal contents.

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