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Novel *Campylobacter lari*-like bacteria from humans and molluscs: description of *Campylobacter peloridis* sp. nov., *Campylobacter lari* subsp. *concheus* subsp. nov. and *Campylobacter lari* subsp. *lari* subsp. nov.

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A polyphasic study was undertaken to clarify the taxonomic position of *Campylobacter lari*-like strains isolated from shellfish and humans. The diversity within the strain collection was initially screened by means of fluorescent amplified fragment length polymorphism analysis and whole-cell protein electrophoresis, revealing the existence of two clusters distinct from *C. lari* and other *Campylobacter* species. The divergence of these clusters was confirmed by phenotypic analysis and by 16S rRNA and *hsp60* gene sequence analysis. Phylogenetic analysis identified *C. lari*, *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter insulaenigrae* as the closest phylogenetic neighbours of both taxa. DNA–DNA hybridizations revealed that one cluster, comprising 10 strains, represented a novel *Campylobacter* species, for which the name *Campylobacter peloridis* sp. nov. is proposed, with 2314BVA^T (=LMG 23910^T =CCUG 55787^T) as the type strain. The second cluster, comprising six strains, represents a novel subspecies within the species *C. lari*, for which the name *Campylobacter lari* subsp. *concheus* subsp. nov. is proposed, with 2897R^T (=LMG 21009^T =CCUG 55786^T) as the type strain. The description of *C. lari* subsp. *concheus* has the effect of automatically creating the subspecies *Campylobacter lari* subsp. *lari* subsp. nov. (type strain LMG 8846^T=NCTC 11352^T).

The genus *Campylobacter* was created by Sebald & Veron (1963) and has since been extended with species originating from both humans and animals. At the time of writing, the genus comprises 18 species and 6 subspecies with validly published names. *Campylobacter lari* was described by Benjamin *et al.* (1983) and has been isolated from the intestinal contents of seagulls and animals, from river water and from shellfish. In humans, strains have been isolated sporadically from diarrhoeic faeces and from cases of bacteraemia and other extraintestinal infections, in both immunocompetent and immunodeficient patients. The species was originally referred to as the nalidixic-acid-resistant thermophilic *Campylobacter* (NARTC) group, but the existence of biochemical variants has been reported, including urease-producing (so-called UPTC variants), nalidixic-acid-susceptible (NASC) and urease-producing, nalidixic-acid-susceptible (UP-NASC) strains. Endtz *et al.* (1997) observed a striking heterogeneity amongst and within the different groups of *C. lari* variants; this was confirmed by Duim *et al.* (2004), who identified four distinct subgroups using numerical analysis of amplified fragment length polymorphism (AFLP) profiles and of partial protein profiles.

In a long-term study of the diversity of *Campylobacter*-like organisms, we collected *C. lari*-like strains from a wide range of isolation sources and geographical regions. Strain details are provided in Supplementary Table S1, available in IJSEM Online. Our aim was to study the aforementioned diversity and re-evaluate the taxonomic position of these strains, using a polyphasic approach.

Abbreviations: AFLP, amplified fragment length polymorphism; UPGMA, unweighted pair group method with arithmetic means; UPTC, urease-producing thermophilic *Campylobacter*.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 21009^T and LMG 23910^T are AM922330 and AM922331, respectively, and those for the *hsp60* gene sequences of strains LMG 22716^T, LMG 23910^T, LMG 11251, LMG 21009^T and LMG 11760 are AM924135–AM924139, respectively.

A neighbour-joining phylogenetic tree based on *hsp60* gene sequences and a table of strains are available as supplementary data with the online version of this paper.

Diversity within the strain collection was initially assessed using whole-cell protein electrophoresis and AFLP analysis. Selected isolates from the observed clusters were further investigated by means of 16S rRNA and *hsp60* gene sequence analysis and by phenotypic analysis. Finally, DNA–DNA hybridizations were performed between representatives of each novel group and closely related taxa.

Strains were grown on Mueller–Hinton agar, supplemented as necessary with 5 % sterile horse blood, at 37 °C for 24–48 h, after which DNA was extracted as described by Pitcher *et al.* (1989).

AFLP analysis was performed as described by Duim *et al.* (1999). 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. Primers complementary to the adaptor and restriction site sequence were used in subsequent pre-selective and selective PCR amplification reactions. The amplified and fluorescently labelled fragments were loaded on a denaturing polyacrylamide gel on an ABI Prism 377 automated sequencer (Applied Biosystems). GeneScan 3.1 (Applied Biosystems) was used for data collection, and the profiles generated were imported, using the CrvConv filter, in BioNumerics 4.61 (Applied Maths) for normalization and further analysis. Similarity between normalized profiles was determined using Pearson's product–moment correlation coefficient and a dendrogram was constructed using the unweighted pair group method with arithmetic means (UPGMA) algorithm. Numerical analysis differentiated two groups of *C. lari*-like organisms distinct from the classical *C. lari* and *C. lari* UPTC clusters, with different clusters exhibiting less than 35 % similarity. The *C. lari* UPTC reference strains were selected from previous studies (Duim *et al.*, 2004; On & Harrington, 2000; Vandamme *et al.*, 1991) and formed two distinct clusters, confirming previous reports on the extensive genetic diversity within this group (Matsuda *et al.*, 2003; On & Harrington, 2000). The taxonomy of the *C. lari* UPTC strains is the subject of a separate study (C. Fitzgerald, L. O. Helsel, A. Steigerwalt, P. Vandamme, J. Pruckler, M. Daneshvar, M. A. Nicholson, C. S. Harrington, P. I. Fields and S. L. W. On, unpublished results). Reference strains from *Campylobacter jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *Campylobacter coli* and *Campylobacter insulaenigrae*, the closest phylogenetic neighbours of *C. lari* on the basis of 16S rRNA gene sequence analysis, could be differentiated from all *C. lari*-like strains examined (Fig. 1). Clusters I (n=10) and II (n=6) correspond to genogroups IV and III, respectively, as described by Duim *et al.* (2004).

For whole-cell protein SDS-PAGE, isolates were grown on Mueller–Hinton agar supplemented with 5 % sterile horse blood and incubated at 37 °C for 48 h under microaerobic conditions ($O_2/CO_2/H_2/N_2$, approx. 4 : 6.5 : 6.5 : 83). The

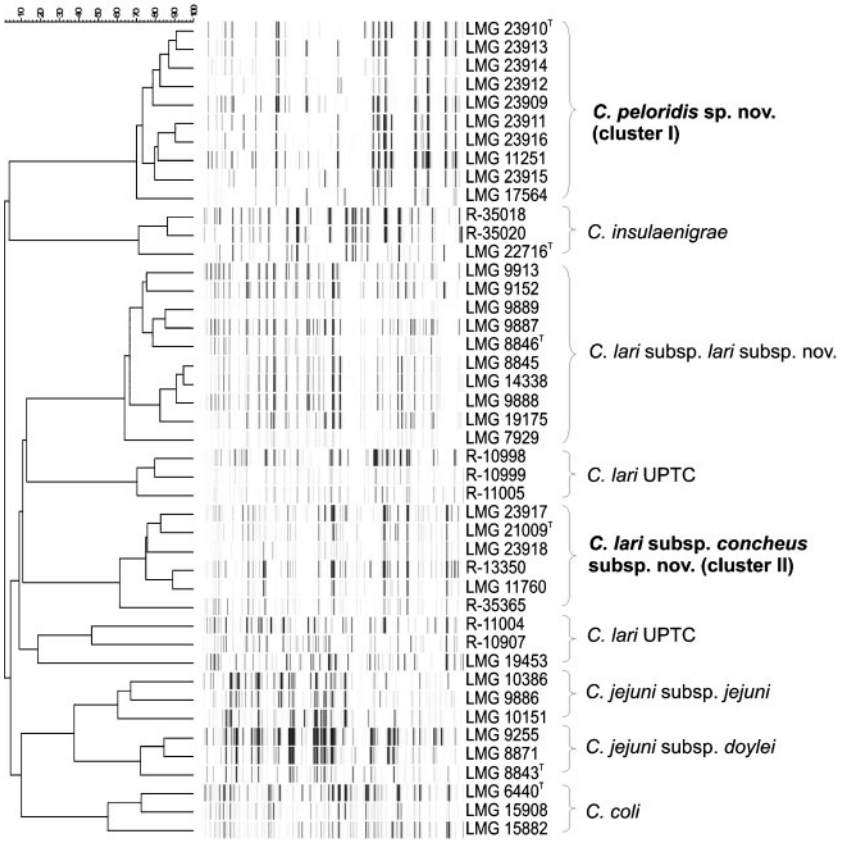

Fig. 1. UPGMA dendrogram of AFLP profiles.

preparation of whole-cell proteins and SDS-PAGE were performed as described by Pot *et al.* (1994). Normalization of densitometric traces was performed using GelCompar 4.2 (Applied Maths). Numerical analysis was performed using Pearson's product–moment correlation coefficient and UPGMA with BioNumerics 4.61 software. For numerical analysis, only the region comprising approx. 20–35 kDa was used, as this part showed additional heterogeneity, which was also described by Duim *et al.* (2004). Cluster I, as observed in the AFLP analysis, was also observed in the protein analysis and was divergent from the classical *C. lari* cluster (83 % similarity). Cluster II could also be discriminated from the classical *C. lari* cluster, but at a higher similarity level (88 %) (Fig. 2). The *C. lari*-like strains could also be differentiated from *C. jejuni*, *C. coli* and *C. insulaenigrae* reference strains (data not shown).

To support the delineation of the groups defined by the above genomic and proteomic analyses, phenotypic testing was performed using tests known to reveal variation among *C. lari* strains (On *et al.*, 1996; S. W. L. On, unpublished results). These included evaluation of growth on media containing 2.0 % NaCl, 1.0 % glycine, 0.05 % safranin, nalidixic acid (32 mg l^{-1}), cephalothin (32 mg l^{-1}), metronidazole (4 mg l^{-1}), carbenicillin (32 mg l^{-1}) and 0.1 % sodium deoxycholate. Growth on unsupplemented *Campylobacter* charcoal deoxycholate agar, growth on unsupplemented nutrient agar (no. 2; Oxoid) and the presence of urease activity were also evaluated. The methods used for biochemical testing were as described previously (On & Holmes, 1991a, b, 1992). Characteristics that served to differentiate clusters I and II from each other and from other *Campylobacter* species are listed in Table 1.

The phylogenetic positions of a selection of isolates from clusters I (LMG 23910^T and LMG 11251) and II (LMG 21009^T and LMG 11760) were determined by analysing almost-complete 16S rRNA gene sequences. Amplification, purification and sequencing of the 16S rRNA genes were performed as described by Vandamme *et al.* (2006). Sequence assembly was performed using BioNumerics 4.61 and selected sequences were aligned using CLUSTAL_X. Subsequently, the aligned sequences were imported into BioNumerics for phylogenetic analyses and bootstrap analysis (500 replicates). Ambiguous bases were discarded for the analysis and a rooted phylogenetic tree was constructed using the neighbour-joining method, with *Caminibacter hydrogeniphilus* AM1116^T as an outgroup (Fig. 3). The tree mainly comprises the closely related, thermotolerant *Campylobacter* species; a full phylogenetic tree for the genus *Campylobacter* was published recently (Debruyne *et al.*, 2008). The 16S rRNA gene sequence similarity between strains LMG 23910^T and LMG 11251 was 100 %, while that between strains LMG 21009^T and LMG 11760 was 99.9 %. The similarity between the 16S rRNA gene sequences of LMG 23910^T and LMG 21009^T was 97.6 %. Comparison with sequences available in the EMBL database indicated that, for both clusters, the closest phylogenetic neighbours (showing >97 % sequence similarity) included *C. lari*, *C. jejuni*, *C. coli* and *C. insulaenigrae*.

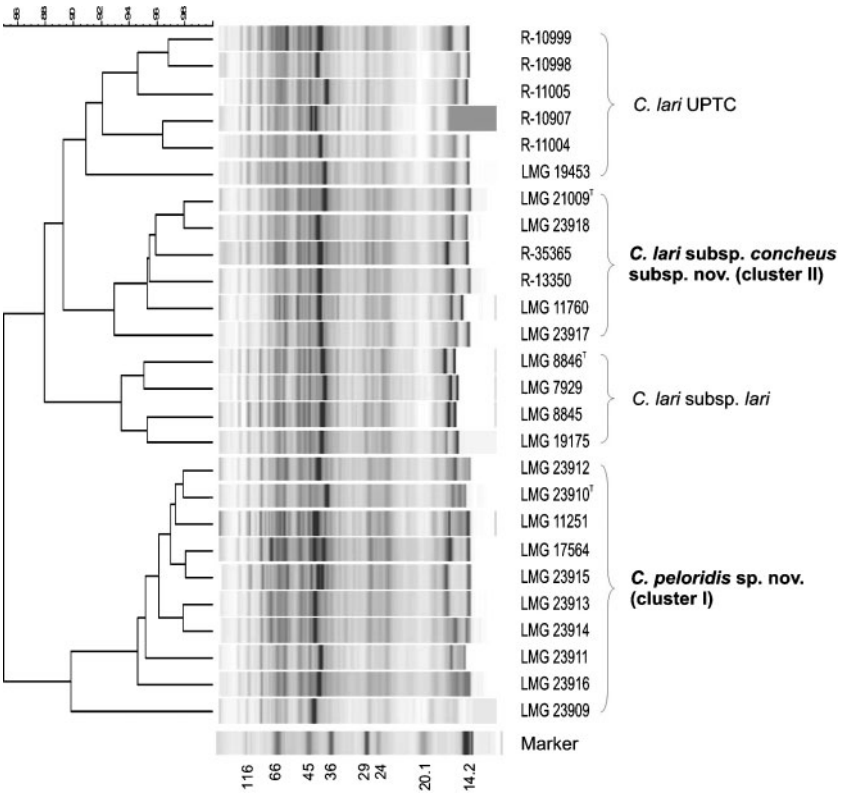

Fig. 2. UPGMA dendrogram of partial whole-cell protein SDS-PAGE profiles. The molecular mass markers used were β-galactosidase (116 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.2 kDa).

Table 1. Differential phenotypic characteristics of the novel strains and related *Campylobacter* species

Taxa: 1, *C. peloridis* sp. nov. (cluster I; *n*=10); 2, *C. lari* subsp. *concheus* subsp. nov. (cluster II; *n*=4); 3, *C. lari* subsp. *lari* subsp. nov; 4, *C. insulaenigrae*; 5, *C. canadensis*; 6, *C. coli*; 7, *C. concisus*; 8, *C. curvus*; 9, *C. fetus* subsp. *fetus*; 10, *C. fetus* subsp. *venerealis*; 11, *C. gracilis*; 12, *C. helveticus*; 13, *C. hyointestinalis*; 14, *C. hominis*; 15, *C. jejuni*; 16, *C. lanienae*; 17, *C. mucosalis*; 18, *C. rectus*; 19, *C. showae*; 20, *C. sputorum*; 21, *C. upsaliensis*. +, All strains positive; −, all strains negative; (+), 80–94 % strains positive; (−), 5–24 % strains positive; V, 40–67 % strains positive; ND, no data available. Additional data for reference species were taken from Inglis *et al.* (2007), Lawson *et al.* (2001) and On *et al.* (1996).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Catalase activity	+	+	+	+	V	+	−	−	+	(+)	(−)	−	+	−	(+)	+	−	(−)	+	V	+
Hippurate hydrolysis	−	−	−	−	−	−	−	(−)	−	−	−	−	−	−	+	−	−	−	−	−	−
Growth at/with/in:																					
42 °C	+	+	+	−	+	+	(+)	V	(+)	−	(−)	+	+	V	V	+	+	(−)	V	(+)	+
NaCl (2 %)	(+)	+	+	−	ND	−	(−)	V	−	−	(−)	(−)	−	V	−	−	(+)	V	+	+	−
Nutrient agar	+	+	+	V	−	+	(−)	+	+	(+)	+	(+)	+	+	+	−	+	(−)	V	(+)	+
Glycine (1 %)	+	+	+	−	V	(+)	(−)	+	+	(−)	+	V	V	+	V	−	V	+	V	+	+
Safranin (0.05 %)	−	−	+	−	ND	+	(−)	+	+	(+)	+	−	+	−	V	−	+	−	−	(+)	+
Sodium deoxycholate (0.1 %)	V	V	+	+	ND	+	(−)	(+)	+	(+)	(+)	(−)	V	−	V	−	−	−	−	V	V
Nalidixic acid (32 mg l^{-1})	(+)	−	(+)	−	V	−	(+)	+	+	V	V	−	+	(+)	−	+	(+)	(+)	−	(+)	−
Cephalothin (32 mg l^{-1})	(−)	+	+	+	−	+	−	−	−	−	−	−	(−)	−	V	+	V	−	−	−	(−)
Metronidazole (4 mg l^{-1})	+	+	+	+	ND	(+)	(−)	−	(+)	V	−	V	V	−	V	+	(+)	−	+	(−)	(+)
Carbenicillin (32 mg l^{-1})	−	+	+	+	ND	(+)	−	−	−	−	−	V	−	−	V	+	−	−	−	−	−

Phylogenetic relationships were further examined using sequencing of the *hsp60* gene (also known as *cpn60* or *groEL*). The *hsp60* gene encodes the 60 kDa chaperonin protein that is found in virtually all members of the *Bacteria*; the usefulness of this target for phylogenetic analysis is well established (Kärenlampi *et al.*, 2004). Kärenlampi *et al.* (2004) introduced phylogenetic analysis based on the *hsp60* gene for the genus *Campylobacter*. By cloning and sequencing a PCR-amplified fragment, they obtained a phylogenetic tree with topology similar to that of 16S rRNA gene sequence-based trees, but with increased resolution due to lower interspecies similarities in the *hsp60* gene. The use of the *hsp60* gene as a phylogenetic marker for the genus *Campylobacter* was further optimized by the implementation of direct sequencing of PCR-amplified *hsp60* gene sequences (Hill *et al.*, 2006).

The reaction mixture and cycling conditions for the PCR were optimized in the present study. The PCR amplification reactions contained 1 × PCR buffer, 200 μM dNTPs,

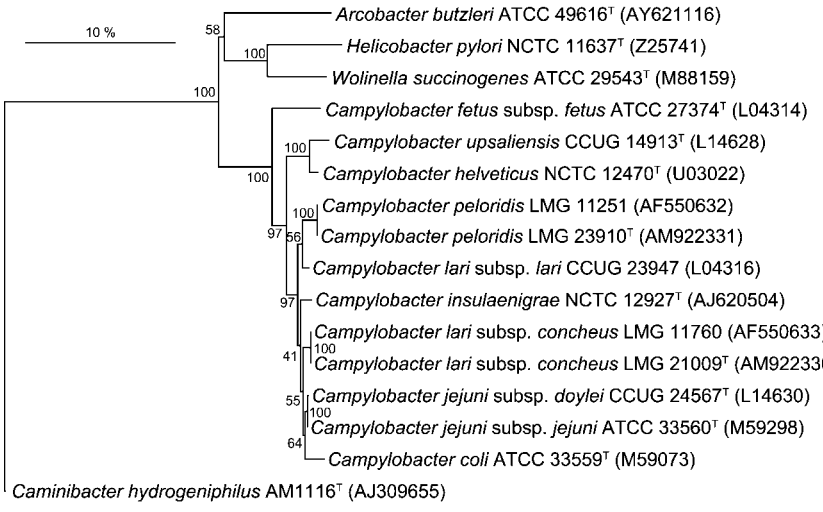

Fig. 3. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, for selected species. Bootstrap percentages (based on 500 replicates) are indicated at nodes. Bar, 10 % sequence divergence.

0.5 U *Taq* polymerase, 5 pmol forward primer H729 (5′-CGCCAGGGTTTTCCCAGTCACGACGAIIIIGCIGGI-GAYGGIACIACIA-3′), 5 pmol reverse primer H730 (5′-AGCGGATAACAATTTCACACAGGAYKIYKITCICCRAA-ICCIGGIGCYTT-3′) and 25 ng genomic DNA, with the final volume adjusted to 25 μl. The amplification primers include landing sites for the sequencing primers (underlined), enabling direct sequencing (Hill *et al.*, 2006). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 2 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 46 °C for 30 s and elongation at 72 °C for 30 s and a final elongation step at 72 °C for 5 min. Sequencing and further analysis were performed as described for 16S rRNA gene sequencing. Comparison of partial *hsp60* gene sequences (555 bp) with sequences in the EMBL database, using the FASTA algorithm, revealed that, for cluster I strains LMG 23910^T and LMG 11251, the closest neighbour was *C. lari* (88–90 %). For cluster II strains LMG 21009^T and LMG 11760, the closest phylogenetic neighbour was again *C. lari*, though higher similarity levels were observed (>96 %), followed by *C. jejuni* and *C. coli* (<89 %). A rooted neighbour-joining tree, with *Arcobacter butzleri* ATCC 49616^T as an outgroup, representing the *hsp60* gene sequence phylogeny, is presented in Supplementary Fig. S1.

For the determination of G+C content, DNA was enzymically degraded into nucleosides as described by Mesbah & Whitman (1989). The nucleoside mixture was separated using HPLC with 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 DNA G+C contents of strains LMG 23910^T (cluster I) and LMG 21009^T (cluster II) were 29 and 30 mol%, respectively. These values are within the range (29–47 mol%) reported for the genus *Campylobacter*.

DNA–DNA hybridizations were performed between strains LMG 23910^T (cluster I) and LMG 21009^T (cluster II) and the type strains of the four most closely related species, i.e. *C. lari*, *C. jejuni*, *C. coli* and *C. insulaenigrae*, and between LMG 21009^T and LMG 23910^T. DNA was extracted from 0.25–0.5 g (wet weight) cells as described by Pitcher *et al.* (1989). DNA–DNA hybridizations were performed with photobiotin-labelled probes in microplate wells (Ezaki *et al.*, 1989), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 30 °C. Reciprocal experiments were performed for every pair of strains; the standard deviations ranged from 0.8 to 9.0 %. DNA–DNA binding values between strain LMG 23910^T (cluster I) and the type strains of *C. lari* (LMG 8846^T), *C. jejuni* (LMG 8841^T), *C. coli* (LMG 6440^T) and *C. insulaenigrae* (LMG 22716^T) were 45, 22, 15 and 33 %, respectively. The DNA–DNA binding values between strain LMG 21009^T (cluster II) and the type strains of *C. lari*, *C. jejuni*, *C. coli* and *C. insulaenigrae* were 78, 23, 19 and 35 %, respectively. The DNA–DNA binding value between LMG 23910^T and LMG 21009^T was 51 %. For LMG 23910^T, the values are well below the threshold (70 %) suggested for species delineation (Stackebrandt & Goebel, 1994), but the results for LMG 21009^T and LMG 8846^T indicate that these strains represent the same species. This confirms preliminary results from hybridization experiments performed between strain LMG 11760 (cluster II) and LMG 8846^T (Duim *et al.*, 2004). Both the genotypic and phenotypic analyses allowed us to distinguish these taxa from established *Campylobacter* species. Therefore we propose that the cluster I strains represent a novel species, for which we propose the name *Campylobacter peloridis* sp. nov., and that the cluster II strains represent a novel subspecies within *C. lari*, for which we propose the name *Campylobacter lari* subsp. *concheus* subsp. nov. With reference to data from previous studies (Benjamin *et al.*, 1983; On *et al.*, 1996), we correspondingly emend the species description of *C. lari* and propose *Campylobacter lari* subsp. *lari* subsp. nov. to encompass the classical nalidixic-acid-resistant, urease-negative isolates typified by the type strain. The divergence of the novel taxa presented here has been confirmed in previous studies, including a multilocus sequence typing system analysis (Miller *et al.*, 2005) and a phylogenetic analysis of a partial GTPase-encoding gene (van Doorn *et al.*, 1998).

Emended description of *Campylobacter lari* Benjamin *et al.* 1984

Cells are Gram-negative, spiral to curved rods, 0.3 × 1.7–2.4 μm in size. After 72 h culture under microaerobic conditions on 5 % blood agar, colonies are colourless, round, entire, convex and 1–1.5 mm in diameter. Cells show rapid darting motility by means of single bipolar flagella. Oxidase- and catalase-positive. Strains do not hydrolyse hippurate. Under microaerobic conditions, strains grow at 37 and 42 °C but not at 25 °C. No growth occurs in air at either 25 or 37 °C. Acid from glucose is not detected. Growth under microaerobic conditions is observed on unsupplemented *Campylobacter* charcoal deoxycholate agar and on blood agar media containing 1.0 % glycine, 2.0 % NaCl and 32 mg carbenicillin l^{-1}. Strains have been isolated from cases of human diarrhoea and bacteraemia, from horse intestine, from the faeces of wild birds, dogs and chickens, from environmental water samples and from shellfish.

Description of *Campylobacter lari* subsp. *lari* subsp. nov.

Campylobacter lari subsp. *lari* (la′ri. L. gen. n. *lari* of a gull).

Strains conform to the species description given above and also exhibit the following characteristics. Growth occurs on media containing 0.1 % trimethylamine *N*-oxide under anaerobic conditions. Strains do not grow on casein medium or on media containing 0.02 % pyronin under microaerobic conditions. Urease- and DNase-negative. Nitrate is reduced. α-Haemolysis is observed on 5 % blood

agar. Microaerobic growth occurs on unsupplemented nutrient agar, on nutrient agar media containing 0.02 or 0.05 % safranin and 0.1 % sodium deoxycholate and on blood agar media containing 4 mg metronidazole l^{-1}, 32 mg carbenicillin l^{-1} and 64 mg cefoperazone l^{-1}. Strains have been isolated from cases of human diarrhoea and bacteraemia, from horse intestine and from the faeces of wild birds, dogs and chickens.

The type strain, LMG 8846^T (=NCTC 11352^T), was isolated from gull faeces in 1976.

Description of *Campylobacter lari* subsp. *concheus* subsp. nov.

Campylobacter lari subsp. *concheus* (con′che.us. L. masc. adj. *concheus* of, or pertaining to, shellfish).

Strains conform to the species description given above. Strains of this subspecies can be distinguished from *C. lari* subsp. *lari* by their inability to grow on media containing 0.05 % safranin. Growth on media containing 0.1 % sodium deoxycholate is strain dependent. Pathogenicity unknown.

Strains have been isolated from human faeces and from shellfish. The type strain, 2897R^T (=LMG 21009^T =CCUG 55786^T), was isolated from shellfish in 1993.

Description of *Campylobacter peloridis* sp. nov.

Campylobacter peloridis (pe′lo.ri.dis. L. gen. n. *peloridis* of a large shellfish, of the giant mussel).

Cells are slightly curved, Gram-negative rods. Colonies are colourless, round, entire, convex and 1–1.5 mm in diameter after culture on 5 % blood agar for 72 h under microaerobic conditions. Oxidase- and catalase-positive. Growth occurs on media containing 1.0 % glycine and 4 mg metronidazole l^{-1}. Most known strains (8/10) grow on media containing 2 % NaCl or 32 mg nalidixic acid l^{-1}. Most known strains (9/10) do not grow on media containing 32 mg cephalothin l^{-1}. Strains do not grow on media containing 0.05 % safranin or 32 mg carbenicillin l^{-1}. Pathogenicity is unknown.

Strains have been recovered from human faeces, from dialysis fluid and from shellfish. The type strain, 2314BVA^T (=LMG 23910^T =CCUG 55787^T), was isolated from shellfish in 1993.

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