Mannheimia caviae sp. nov., isolated from epidemic conjunctivitis and otitis media in guinea pigs

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Strains T138021-75^T, Pg19 and Pg20 (taxon 25 of Bisgaard) were isolated from guinea pigs and characterized. Strains T138021-75^T and Pg20 showed identical 16S rRNA gene sequences and were distantly related to the published strain P224 with the highest 16S rRNA similarity of 98.6%. These two strains showed 97.8% sequence similarity with the type strain and other strains of *Mannheimia glucosida* and 97.3% similarity with the type strain of *Mannheimia varigena*, but <97% similarity with all other type strains of the genus *Mannheimia*, including *Mannheimia haemolytica* (96.9%). Phylogenetic analysis of *rpoB* gene sequences showed that strain P224 had a distant position (89.9% gene sequence similarity) compared with the three other strains (T138021-75^T, Pg20 and Pg19), which had identical gene sequences. These three novel strains also shared identical *recN* gene sequences. Phylogenetic analysis of the *recN* gene sequences showed a close relationship between the three novel strains and strain P224. The DNA–DNA reassociation value between strain T138021-75^T and P224 was 81.6% and 40.3% between strain T138021-75^T and the type strain of *M. glucosida*. Based on the DNA–DNA reassociation data, strain T138021-75^T belonged to a separate species that was closely related to strain P224. Strain P224 differed from strains T138021-75^T, Pg20 and Pg19 in the following phenotypic characteristics: activity of ornithine carboxylase, hydrolysis of glycosides, and acid formation from maltose, dextrin, melibiose and raffinose, as well as reactions for α -galactosidase and β -xylosidase. Whole genome similarity calculations based on *recN* gene sequences showed that strains T138021-75^T and P224 were related at the species level (0.932), whereas 16S rRNA and partial *rpoB* gene sequence comparisons showed a more divergent position of strain P224 compared with the novel strains, including a different host of isolation. The results showed that the three strains of taxon 25 represent a novel species for which the name *Mannheimia caviae* sp. nov. is proposed. The type strain, T138021-75^T (=CCUG 59995^T=DSM 23207^T) was isolated from purulent conjunctivitis in guinea pigs. Previous publications have documented both ubiquinones and demethylmenaquinone to be present in the type strain. The G + C content of the DNA of the type strain has been found to be 41.4 mol% (T_m).

Although they have been known for decades, the taxonomy of most taxa of the family *Pasteurellaceae* that are associated with laboratory rodents has remained unsolved (Bisgaard, 1993; Christensen & Bisgaard, 2008). An unclassified *Pasteurella*-like organism was reported to be associated with subdermal abscesses in guinea pigs by Stewart & Letscher (1976). Very similar isolates were subsequently reported as the 'SP' group by Frederiksen (1981). Several phenotypically identical *Pasteurella*-like organisms were also reported by Mannheim *et al.* (1978) from an outbreak of epidemic

conjunctivitis in a large colony of guinea pigs. Although widely distributed among mammals and birds, infections due to *Pasteurella multocida* do not appear to be common in guinea pigs (Ganaway, 1976). Isolates belonging to *P. multocida* biovar 1 were, however, reported by Frederiksen (1973). Mráz *et al.* (1979) demonstrated that [*Pasteurella*] *ureae* was present in 47.5% of guinea pigs investigated. This species was later reclassified and named as *Actinobacillus ureae* (Mutters *et al.*, 1986). Attempts to clarify the natural occurrence of members of the family *Pasteurellaceae* in two colonies of conventional and well managed guinea pigs by Bisgaard *et al.* (1983), demonstrated at least four novel taxa (originally termed Bisgaard Taxa 5 to 8), all of which have subsequently been demonstrated to represent genuine members of the family *Pasteurellaceae* (Christensen & Bisgaard, 2008).

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA, *rpoB* and *recN* gene sequences of strain T138021-75^T are HM439607, HM439605 and HM439606, respectively.

Supplementary figures and a supplementary table are available with the online version of this paper.

Organisms tentatively designated as members of the SP-group have been reported from guinea pigs and from human blood and faeces (Frederiksen, 1981). Guinea pig isolates classified as belonging to the SP-group or taxon 6, respectively, give clearly different results in both biochemical (Boot & Bisgaard, 1995) and serological tests (Boot *et al.*, 1995). Based upon 41 phenotypical characteristics, Kunstýr & Hartmann (1983) investigated the prevalence of members of the family *Pasteurellaceae* in laboratory animals, including guinea pigs. Unfortunately, information on the hosts associated with the different taxa demonstrated was not provided. Although a final classification was not given, Boot *et al.* (1983) concluded that members of the *Pasteurella*–*Actinobacillus* group must be considered as potentially pathogenic for guinea pigs. Subsequently, Boot & Walvoort (1986) concluded that otitis media, from which members of the family *Pasteurellaceae* also could be demonstrated, must be considered a major disease problem in guinea pigs. The importance of including reference or type strains for comparison and extended phenotypic characterization to avoid misclassification was demonstrated by Boot & Bisgaard (1995). Isolates from guinea pigs previously reported as *Pasteurella gallinarum* (later reclassified as *Avibacterium gallinarum*) were reclassified as members of the SP-group, taxon 6 or taxon 25. Two isolates, Pg19 and Pg20, recovered from guinea pigs suffering from otitis media and classified as taxon 25, demonstrated phenotypical characteristics that were clearly different from the other species of the family *Pasteurellaceae* that were also studied (Boot & Bisgaard, 1995). These two isolates have subsequently been shown to share the phenotypical characteristics of a group of organisms represented by strain T138021-75^T as reported by Mannheim *et al.* (1978) (M. Bisgaard, unpublished data). The aims of the present study were to classify and name organisms tentatively named taxon 25 in order to improve the diagnosis of these organisms, gain a better understanding of their epidemiology and enable the selection of suitable strains for virulence studies.

The three clinical isolates, strains T138021-75^T, Pg19 and Pg20, were characterized. Strain P224 (see Supplementary Table S1 available in IJSEM Online), previously identified as being a possible species of the genus *Mannheimia* (Kuhnert *et al.*, 2007), was also included for comparison. The type strains of the five species of the genus *Mannheimia* (Angen *et al.*, 1999) and the type strain of *P. multocida* were also included for comparison based on their published DNA sequences. Phenotypic characterization was carried out as reported previously (Bisgaard *et al.*, 1991; Christensen *et al.*, 2007) which enabled comparisons to be made with previous characteristics determined in our laboratory. For phylogenetic comparisons of 16S rRNA and *rpoB* gene sequences, additional sequences of *Mannheimia haemolytica* and *Mannheimia glucosida* were included based on the highest score with a BLAST search (Altschul *et al.*, 1997) in GenBank (Benson *et al.*, 2006).

The partial *rpoB* gene sequences of the three isolates investigated (Supplementary Table S1) were determined

according to Mollet *et al.* (1997) covering the region 509–680 (positions according to *Escherichia coli* numbering) of the deduced protein sequence as reported previously (Angen *et al.*, 2003; Korczak *et al.*, 2004). Sequencing of the 16S rRNA gene of two strains (Supplementary Table S1) was performed according to previous reports (Christensen *et al.*, 2002a; Angen *et al.*, 2003). In addition, *recN* gene sequences were used as a representative target for determining whole-genome sequence similarity as described by Kuhnert & Korczak (2006), with 1340 bp of the gene being sequenced. Primers recN-L and recN-R were used for PCR and for sequencing the additional primers recNR + recNMannF (5'-AGCAATCTCGCTTGCTCGTA) and recNL + recNTx 25R1 (5'-AAAGCTCGGTCAGTTCTTCG) were included. Sequencing was performed by Macrogen (Korea). For *recN* gene sequences, whole genome similarity values were calculated according to Zeigler (2003). Pairwise comparisons for similarity were performed by the program WATER included in EMBOSS (Rice *et al.*, 2000). Multiple alignment was performed with CLUSTAL_X (Thompson *et al.*, 1997). Maximum-likelihood analysis including bootstrap analysis was performed by fastDNAmI (Olsen *et al.* 1994; Felsenstein, 1995) on a Linux compatible server. The analysis was run with transition/transversion ratios of 1.3, 2.4 and 1.8 for the 16S rRNA, *rpoB* and *recN* multiple gene sequence alignments, respectively.

Phylogenetic analysis of nearly full-length 16S rRNA gene sequences, partial *rpoB* gene sequences and nearly full-length *recN* gene sequences are shown in Fig. 1, Supplementary Fig. S1 and Supplementary Fig. S2, respectively. Strains T138021-75^T and Pg20 showed identical 16S rRNA gene sequences and were distantly related to other species of the genus *Mannheimia* including strain P224 (98.6%) (Fig. 1). The highest 16S rRNA gene sequence similarity (97.8%) to other taxa was to the type strain and other strains of *M. glucosida* (P733, P933 and H62). The type strain of *Mannheimia varigena* showed 97.3% 16S rRNA gene sequence similarity. All other type strains of the genus *Mannheimia*, including the type strain of *M. haemolytica* (96.9%), showed less than 97% similarity.

Phylogenetic analysis of the *rpoB* gene sequences showed the distant position of strain P224 compared with the other three strains (T138021-75^T, Pg20, Pg19) that shared identical sequences (Supplementary Fig. S1). The closest related strains included the type strains of *M. varigena* and *M. haemolytica* (biovar 8), both showing 93.9% similarity, while strain P224 only demonstrated 89.9% sequence similarity. The highest *rpoB* gene sequence similarity for strain P224 was 95.1%, which was observed with the type strains of *M. haemolytica* and *M. glucosida*.

The three strains of taxon 25 (T138021-75^T, Pg20 and Pg19) also shared identical *recN* gene sequences. However, here the phylogenetic analysis showed a close relationship with strain P224 (Supplementary Fig. S2). The close relatedness of strain P224 to the three strains of taxon 25

Fig. 1. Phylogenetic relationships between the novel isolates investigated and recognized members of the family *Pasteurellaceae* based on maximum-likelihood analysis of near full-length 16S rRNA gene sequences. Support for monophyletic groups by bootstrap-analysis is indicated as numbers out of 100. Strain numbers are followed by DDBJ/EMBL/GenBank accession numbers. Strain numbers in bold represent strains that were sequenced in the present investigation. Strains in parentheses share sequences with the preceding strain at the same branch. Bar, sequence variation considering the models for nucleotide substitution and tree-shape used in the maximum-likelihood analysis.

in the 16S rRNA and *recN* gene sequence phylogenies, but not in the *rpoB* gene sequence phylogeny, may point to the horizontal gene transfer of *rpoB* from another source other than the last common ancestor of P224 and strains T138021-75^T, Pg19 and Pg20. However, within the family *Pasteurellaceae*, the *rpoB* gene has often been used to infer organism phylogeny and has not previously been suspected as having been transferred between taxa.

A threshold value of 97% 16S rRNA gene sequence similarity resulting in DNA–DNA reassociation values <70% has been suggested as the threshold for species separation from a taxonomically conservative point of view (Tindall *et al.*, 2010). For this reason, DNA–DNA hybridizations were performed between strains T138021-75^T and P224 and between strains T138021-75^T and *M. glucosida* DSM 19638^T by the spectrometric method by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig). Cells were disrupted by using a French pressure cell (Thermo Spectronic) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell charger and a temperature controller with *in situ* temperature probe (Varian). Measurements were performed in 2 × SSC at 66 °C. DNA reassociation values between strains T138021-75^T and P224 were 82.6 and 80.5% (mean 81.6%). DNA relatedness values between strain T138021-75^T and *M. glucosida* DSM 19638^T were 41.8% and 38.7% (mean 40.3%). Based on the DNA–DNA reassociation data, strain T138021-75^T belongs to a separate species to *M. glucosida* when the recommended threshold value of 70% of DNA–DNA reassociation between species is applied (Wayne *et al.*, 1987). Species of the family *Pasteurellaceae* have mainly been defined on the basis of DNA–DNA reassociation values of 80–85% as measured by the spectrophotometric method (Mutters *et al.*, 1989; Christensen *et al.*, 2002b,

2005). This places strain P224 at the borderline compared with strain T138021-75^T.

All three novel strains (T138021-75^T, Pg19 and Pg20) tested positive for catalase and oxidase activities and they were fermentative in Hugh and Leifson medium with (+)-D-glucose. The strains stained Gram-negative and were non-motile pleomorphic rods when observed at 22 and 37 °C. Common characteristics are further listed in the species description. Different reactions between the strains were observed for acid production from (–)-D-arabinose (strain Pg20 late positive), (–)-D-sorbitol (strains Pg19 and Pg20 positive), (–)-L-fucose (strains Pg19 and Pg20 late positive) and α-fucosidase (o-nitrophenyl α-L-fucopyranoside; ONPF) (strains Pg19 and Pg20 negative). The phenotypic characteristics obtained were in accordance with those previously reported for members of the genus *Mannheimia* (Angen *et al.*, 1999). Characteristics that separated the guinea pig isolates from other species of the genus *Mannheimia* are presented in Table 1. The novel strains were separated from other recognized species of the genus *Mannheimia* on the basis of 4–9 different characteristics; the most closely related species was *M. glucosida* (Table 1). Differences in ornithine decarboxylase, growth on MacConkey agar, β-glucosidase, α-galactosidase, β-xylosidase and production of acid from maltose, (+)-melibiose, raffinose, dextrin and glycosides separated the three novel strains from strain P224 (Kuhnert *et al.*, 2007). Characteristics previously reported for strain T138021-75^T (Mannheim *et al.*, 1978) were confirmed in this study.

The G+C content of DNA for strain T138021-75^T was estimated to be 41.4 mol% (*T_m*) (Mannheim *et al.*, 1978), while 41.6 mol% was reported for *M. glucosida* (Angen *et al.*, 1999), its closest relative based upon phenotypic tests, and clearly within the variation of the genus *Mannheimia* (Angen *et al.*, 1999).

The ability to produce ubiquinones in addition to demethylmenaquinones characterizes major groups of the family *Pasteurellaceae*, including species of the genus *Mannheimia* (Mannheim, 1981). Both ubiquinones and

Table 1. Phenotypic characteristics separating *Mannheimia caviae* sp. nov. from recognized species of the genus *Mannheimia*

Taxa: 1, *M. caviae* (T138021-75^T, Pg19 and Pg20); 2, *M. haemolytica*; 3, *M. glucosida*; 4, *M. ruminalis*; 5, *M. granulomatis*; 6, *M. varigena*. +, ≥90 % positive within 1–2 days; (+), ≥90 % positive within 3–14 days; –, ≤10 % positive within 14 days; w, weak reaction; D, 11–89 % positive within 14 days. Data for taxa 2–6 are from Angen *et al.* (1999). All taxa were investigated using the same methods and cultural conditions.

Characteristic	1	2	3	4	5	6
β-Haemolysis (bovine blood)	–	+	+	–	–	+
Ornithine decarboxylase	+	–	D	–	–	D
Acid formation from:						
(+)-L-Arabinose	+	–	D	–	–	+
(+)-D-Xylose	+	+	+	–	D	+
myo-Inositol	(+)/w	D	(+)	–	D	D
(–)-D-Sorbitol	D	+	+	D	+	–
(+)-L-Rhamnose	–	–	–	–	–	D
Maltose	–	+	+	D	+/((+)	D
(+)-Melibiose	+	–	–	–	–	–
β-Glucosidase	+	–	+	–	+	–
Glycosides*	+	–	D	–	D	–
α-Fucosidase	D	+	+	D	–	–
α-Galactosidase	+	–	–	–	–	–
β-Xylosidase	+	–	D	–	D	–

*Glycosides: cellobiose, aesculin, amygdalin, arbutin, gentiobiose and salicin.

demethylmenaquinone were produced by strain T138021-75^T according to Mannheim *et al.* (1978), underlining the classification of the novel strain within the family *Pasteurellaceae*.

Recently, Zeigler (2003) showed that a selection of three genes can be used to predict whole-genome relatedness. Based upon the same genes, *recN*, *thdF* and *rpoA*, Kuhnert & Korcak (2006) validated their use in multilocus sequence analysis (MLSA) to predict the whole-genome DNA–DNA similarity for taxa of the family *Pasteurellaceae*. The results obtained showed that strains of the same species showed similarity values of 0.9 and higher, while species of the same genus had similarity values above 0.4. Similarity values between 0.85 and 0.9 were considered intermediate and could indicate different species or subspecies, as observed for *M. haemolytica*/*M. glucosida* (0.88) or with the two subspecies of *Actinobacillus equuli* (0.87). Threshold similarity values for *recN* alone were comparable with very few discrepancies and this comparison was used in the present investigation. All three strains investigated shared the same *recN* gene sequence that showed 98.2 % similarity with strain P224. Only 78.3–81.3 % similarity was observed for species of the genus *Mannheimia*, which converted to whole genome similarities of 0.932 between strains T138021-75^T and P224 and

0.46–0.62 between strain T138021-75^T and recognized species of the genus *Mannheimia*. Whole-genome similarity values calculated from *recN* gene sequences demonstrated values of 0.46, 0.48, 0.48, 0.53 and 0.63 between strain P224 and the type strains of *Mannheimia granulomatis*, *M. glucosida*, *M. haemolytica*, *Mannheimia ruminalis* and *M. varigena*, respectively (Kuhnert *et al.*, 2007).

In conclusion, strains tentatively named taxon 25 were investigated using a polyphasic taxonomic approach and the results showed excellent correlation with the results obtained from extended phenotypic and phylogenetic investigations as well as with calculated whole-genome similarity values based on *recN* gene sequences. The results obtained clearly showed that taxon 25 represents a novel species of the genus *Mannheimia*, for which the name *Mannheimia caviae* sp. nov. is proposed. It is important to name this taxon of the genus *Mannheimia* since it is of potential clinical importance. Strain P224 was closely related to *M. caviae* sp. nov. as the DNA–DNA relatedness value was at the threshold value of 82 % and whole genome similarity calculations based on *recN* gene sequences showed species level relatedness of (0.932) with strain T138021-75^T. 16S rRNA gene (98.6 %) and partial *rpoB* gene sequence comparisons (89.9 %) showed a more divergent position of strain P224 compared with the novel species. Strain P224 could be differentiated from *M. caviae* sp. nov. on the basis of phenotypic characteristics as it was ornithine carboxylase-negative and negative for the hydrolysis of glycosides. Strain P224 was positive in tests for acid formation from maltose and dextrin, but negative for melibiose and raffinose. Tests for α-galactosidase and β-xylosidase were also negative (Kuhnert *et al.*, 2007). Strain P224 was obtained from a rabbit, but the three strains of *M. caviae* sp. nov. were obtained from guinea pigs.

The differences in hosts and genotypic and phenotypic characteristics between strain P224 and strains T138021-75^T, Pg19 and Pg20 have parallels with *M. varigena*, for which two genotypical populations associated with pig and ruminants, respectively, have been previously demonstrated (Angen *et al.*, 1999) and with *M. granulomatis* for which three populations have been documented associated with leporine, bovine and cervine hosts, respectively (Bojesen *et al.*, 2007). Hopefully isolates related to strain P224 will be obtained in future studies to enable more taxonomic information to be generated in order to decide whether this strain belongs to a taxon that can be classified as a separate species, closely related to *M. caviae* sp. nov., or alternatively as a novel subspecies of *M. caviae* sp. nov.

Description of *Mannheimia caviae* sp. nov.

Mannheimia caviae sp. nov. (ca'vi.ae. N.L. n. *Cavia* generic name of the guinea pig; N.L. gen. n. *caviae* of *Cavia*, of a guinea pig).

Cells are non-motile at 22 and 37 °C and are Gram-negative-staining, coccoid or pleomorphic rods. Colonies on bovine blood agar are regular, circular and slightly

raised 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 24 h incubation at 37 °C. β -Haemolysis is not observed on calf blood agar. CAMP negative on calf blood agar [CAMP is an abbreviation of the first initials of family names of the authors who described the test (Christie *et al.*, 1944)]. The consistency of the colonies is unguent-like and the colonies do not adhere to the agar surface. All strains tested are positive for catalase and oxidase activities and demonstrate a fermentative reaction in Hugh and Leifson medium with (+)-D-glucose. Strains give a positive result in tests for porphyrin, nitrate reduction, alanine aminopeptidase, ornithine decarboxylase, phosphatase, growth on MacConkey agar, ONPG (o-nitro-phenyl-D-galactopyranoside), β -glucosidase (p-nitrophenyl β -D-glucopyranoside; NPG), α -galactosidase, β -xylosidase and production of acid from glycerol, (+)-L-arabinose, (–)-D-ribose, (+)-D-xylose, myo-inositol, (–)-D-mannitol, (–)-D-fructose, (+)-D-galactose, (+)-D-glucose, cellobiose, lactose, (+)-melibiose, sucrose, raffinose, aesculin, amygdalin, arbutin, gentiobiose and salicin. Symbiotic growth (NAD requirement) is not observed. The Simmons citrate test is negative, as well as the formation of acid from mucate, base formation from malonate, H₂S/TSI, growth in the presence of KCN, methyl red test, Voges–Proskauer test at 37 °C, production of gas from nitrate, urease, arginine dihydrolase, lysine decarboxylase, phenylalanine deaminase, indole, gelatinase, hydrolysis of Tween 20 and 80, formation of pigment, α -glucosidase (p-nitrophenyl α -D-glucopyranoside; PNPG), β -glucuronidase (p-nitrophenyl β -D-glucopyranosiduronic acid; PGUA), α -mannosidase, production of acid from meso-erythritol, adonitol, (+)-D-arabitol, xylitol, (–)-L-xylose, dulcitol, (+)-D-fucose, (+)-D-mannose, (+)-L-rhamnose, (–)-L-sorbose, maltose, trehalose, (+)-melezitose, dextrin, (+)-D-glycogen, inulin, (+)-turanose, β -N-CH₃-glucosamide and production of gas from (+)-D-glucose. Variations are observed as to acid production from (–)-D-sorbitol and α -fucosidase, the type strain is negative and positive, respectively, for these tests. Acid formation from (–)-D-arabinose is negative for the type strain, but strain Pg20 gives a late positive reaction. Acid formation from (–)-L-fucose is negative for the type strain, but strains Pg19 and Pg20 give a late positive result. All isolates conform to the phenotypic characteristics as described for the genus *Mannheimia* (Angen *et al.*, 1999). These characteristics allow the species to be separated from other species of the genus *Mannheimia* by at least four characteristics. The detailed phenotypic characteristics for all three isolates are presented in this study. Both ubiquinones and demethylmenaquinone are present in the type strain according to Mannheim *et al.* (1978).

The type strain, T138021-75^T (=CCUG 59995^T=DSM 23207^T) was obtained from purulent conjunctivitis in guinea pigs. The G + C content of DNA of the type strain is 41.4 mol % (*T_m*) (Mannheim *et al.*, 1978). Other strains are Pg19 and Pg20.

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