Moraxella porci sp. nov., isolated from pigs

A. I. Vela,^{1,2} C. Sánchez-Porro,³ V. Aragón,⁴ A. Olvera,⁴ L. Domínguez,^{1,2}
A. Ventosa³ and J. F. Fernández-Garayzábal^{1,2}

Correspondence

J. F. Fernández-Garayzábal
garayzab@vet.ucm.es

¹Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense, Madrid, Spain

²Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain

³Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain

⁴Centre de Recerca en Sanitat Animal (CRESA), Campus de Bellaterra-Universitat Autònoma de Barcelona, 08193-Bellaterra, Barcelona, Spain

Nine Gram-negative, catalase- and oxidase-positive, coccus-shaped bacteria were isolated from pigs affected by different pathological processes. Phenotypic and genotypic methods were adopted to determine the relationships of these new isolates to recognized species of the genus *Moraxella*. Analysis of the 16S rRNA gene sequences demonstrated that the clinical isolates represented a new lineage within the genus *Moraxella*. The isolates were closely related to *Moraxella cuniculi* and *Moraxella pluranimalium* with 16S rRNA gene sequence similarities of 98.1 % and 99.1 %, respectively. The isolates displayed DNA–DNA relative binding ratios of 74 % to each other, but distinctly lower levels of DNA–DNA hybridization were observed with phylogenetically closely related moraxellae (<32 %). The new isolates could be distinguished from all other recognized species of the genus *Moraxella* by physiological and biochemical tests. On the basis of the phenotypic and molecular data, the nine new isolates from pigs represent a novel species within the genus *Moraxella*, for which the name *Moraxella porci* sp. nov. is proposed. The type strain is SN9-4M^T (=CECT 7294^T=CCUG 54912^T).

The genus *Moraxella* currently comprises 21 species with validly published names (<http://www.bacterio.net>; Juni & Bøvre, 2005), some of which have been described during the last decade (Angelos *et al.*, 2007; Vela *et al.*, 2009). The recognition of these novel species has been made possible by the use of improved taxonomic methods, such as chemotaxonomic- and molecular-based approaches, especially 16S rRNA gene sequencing. Although some of the members of the genus *Moraxella* have been isolated from animal sources, most of them are usually found as part of the indigenous microbiota of animals (Kodjo *et al.*, 1995, 1997; Juni & Bøvre, 2005) and are not considered of clinical relevance in veterinary medicine. In fact, only *Moraxella bovis* is a well-established animal pathogen, being responsible for ocular and respiratory infections (Brown *et al.*, 1998; Quinn *et al.*, 1999; Lavin *et al.*, 2000). In this study, we characterized nine *Moraxella*-like organisms isolated from different clinical specimens from pigs during routine microbiological diagnosis.

Unidentified Gram-negative coccus-shaped organisms were isolated from different clinical specimens of nine pigs

located in seven farms. Isolates 466-01 and 1335-02 were isolated from the pleura of two pigs with pleuritis. Isolates 1407-02 and 91-02 were isolated from the lungs of two pigs with pneumonia. Isolates 43-02, 464-01 and N148-05CP-52G were isolated from the pericardium of three pigs affected by pericarditis. Isolate SN9-4M^T and isolate 1008-04 were from the brain and an inguinal lymph node of two pigs with meningitis and inguinal adenomegaly, respectively. Samples were collected and frozen at –40 °C until processed in the laboratory. Strains were isolated on Columbia sheep blood agar plates (bioMérieux) after incubation at 37 °C for 24–48 h under aerobic conditions. The new isolates were Gram-stained and assessed for the presence of oxidase and catalase as described by Barrow & Feltham (1993). DNA hydrolysis was tested by using DNase test agar. Growth in brain heart infusion broth was assessed at 4 and 15 °C for up to 14 days, at 22 °C for up to 7 days and at 30, 37 and 42 °C for 48 h. Growth in the presence of 0.5, 1.5, 3, 4 and 6.5 % (w/v) NaCl and under anaerobic (with 4–10 % CO₂) and microaerobic (with 5–15 % O₂ and 5–12 % CO₂) conditions, using GasPak Plus and CampyPak Plus systems (BBL), respectively, was assessed at 37 °C for 48 h. Growth on MacConkey agar (bioMérieux) and chocolate agar (bioMérieux) was tested at 37 °C for 24 and 48 h. Additional biochemical tests were performed by using the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SN9-4M^T is FM872292.

API 20NE, API 20E and API ZYM systems (bioMérieux) and Biolog GN Microplates (Biolog, Inc.) according to the manufacturer's instructions, except that incubation conditions for API 20NE tests were 37 °C for 72 h. Phenotypic studies were also carried out with the reference strains *Moraxella pluranimalium* 248-01^T, *Moraxella cuniculi* CCUG 2154^T, *Moraxella caprae* CCUG 33296^T, *Moraxella bovis* CCUG 2133^T and *Moraxella canis* CCUG 26883^T.

The nine new isolates exhibited identical phenotypic characteristics, except for the assimilation of gluconate (isolate 1335-02 was positive), malate (isolates 1335-02, 1407-02 and 43-02 were negative), methyl pyruvate (isolate SN9-4M^T was negative), monomethyl succinate (isolates 1335-02, 43-02, 1008-04 and N148-05CP-52G were negative) and succinic acid (isolate 1008-04 was negative), growth in the presence of 3 % (w/v) NaCl (isolates 91-02, 466-01, 1008-04 and 1335-02 did not grow) and reduction of nitrate (isolates 43-02 and 1407-02 were negative). A detailed presentation of the morphological, physiological and biochemical characteristics of the isolates is given in the species description and in Table 1.

To establish the phylogenetic affinities of the new isolates, their 16S rRNA gene sequences were determined as described previously (Vela *et al.*, 2005) and subjected to a comparative analysis. The almost complete sequences of the nine new strains were determined and pairwise analysis revealed 99.4–100 % 16S rRNA gene sequence similarity among the isolates. Sequence searches of GenBank using the FASTA program (Pearson, 1994) showed that the new isolates were most closely related to members of the genus *Moraxella*. Strain SN9-4M^T displayed the closest 16S rRNA gene sequence similarity to *M. pluranimalium* 248-01^T (99.1 % sequence similarity) and *M. cuniculi* CCUG 2154^T (98.1 % sequence similarity). These sequences and those of other known related strains were retrieved from GenBank and aligned with the newly determined sequences using the DNATools program (Rasmussen, 1995). Phylogenetic trees were constructed according to three different algorithms: neighbour-joining (Saitou & Nei, 1987), using the DNATools and TreeView programs (Page, 1996), maximum-likelihood, using PHYL software (Guindon & Gascuel, 2003), and maximum-parsimony, using the MEGA (molecular evolutionary genetics analysis) version 3.1 software package (Kumar *et al.*, 2004). Phylogenetic distances for the neighbour-joining and the maximum-likelihood algorithms were calculated by the Kimura two-parameter method (Kimura, 1980) and close-neighbour-interchange (search level=2, random additions=100) was applied in the maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications). The topology of the phylogenetic trees obtained by using the neighbour-joining and the other two methods revealed a clear affiliation of the new isolates to the genus *Moraxella* and placed them as a separate branch within an intrageneric cluster containing *M. cuniculi* CCUG 2154^T and *M. pluranimalium* 248-01^T, which was supported by a bootstrap resampling value of 99 %.

Table 1. Characteristics that are useful for differentiating the novel strains from phylogenetically closely related species of the genus *Moraxella*

Taxa: 1, *M. porci* sp. nov. (nine strains); 2, *M. pluranimalium* 248-01^T; 3, *M. cuniculi* CCUG 2154^T; 4, *M. bovis* CCUG 2133^T; 5, *M. canis* CCUG 26883^T; 6, *M. caprae* CCUG 33296^T. Phenotypic data for *M. porci* sp. nov. and for the other species of the genus *Moraxella* were taken from this study. +, Positive; –, negative; c, coccus; r, rod.

Characteristic	1	2	3	4	5	6
Morphology	c	c	c	r	c	r
Tolerance of 3 % NaCl	v*	–	–	+	+	+
Growth at 42 °C	+	–	+	–	+	–
Haemolysis	–	–	–	+	+	+
Nitrate reduction	v*	–	–	+	+	–
Assimilation of:						
Acetic acid	–	–	–	+	+	+
cis-Aconitic acid	–	–	–	+	–	–
Bromosuccinic acid	–	–	+	+	+	+
Citric acid	–	–	–	+	–	–
D-Fructose	–	–	–	+	–	–
Glucose	–	+	–	–	–	–
L-Glutamic acid	–	–	+	+	+	+
Glycogen	–	–	–	+	–	–
L-Histidine	–	–	–	+	–	–
α-Hydroxybutyric acid	–	–	–	+	+	+
β-Hydroxybutyric acid	+	+	+	+	–	+
Inosine	–	–	+	–	–	–
α-Ketobutyric acid	–	+	–	+	–	–
α-Ketovaleric acid	–	–	–	+	–	–
L-Proline	–	+	+	–	–	–
D-Psicose	–	–	–	+	–	–
Putrescine	–	–	+	–	–	–
L-Serine	–	–	–	+	+	+
Succinamic acid	–	–	–	+	–	+
Tween 40	–	–	–	+	+	–
Tween 80	–	–	–	+	+	–
Urocanic acid	–	–	–	–	+	+
Production of:						
Acid phosphatase	–	–	–	–	+	–
Alkaline phosphatase	–	–	–	–	+	–
DNase	–	–	–	–	+	–
Gelatinase	–	–	–	+	–	–
Naphthol-AS-BI phosphohydrolase	+	+	–	–	–	–

*Positive reaction for *M. porci* SN9-4M^T.

The new isolates showed high 16S rRNA gene sequence similarities to each other, although they consistently formed two separate branches when using three different treeing algorithms (Fig. 1). DNA–DNA hybridization experiments were carried out between representative isolates of both branches (SN9-4M^T and 466-01) and between strain SN9-4M^T and its closest phylogenetic neighbours, *M. cuniculi* CCUG 2154^T and *M. pluranimalium* 248-01^T. DNA was extracted and purified by the

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparisons obtained with the neighbour-joining algorithm showing the relationships of the nine isolates of *Moraxella porci* sp. nov. and other reference species of the genus *Moraxella*. Bootstrap values (expressed as a percentage of 1000 replications) >50% are given at the branching points. Solid circles indicate that the corresponding nodes (groupings) are also obtained on the parsimony tree. Open circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood and parsimony trees. *Acinetobacter calcoaceticus* DSM 30006^T (AJ633632) was used as an outgroup. Bar, 1% sequence divergence.

method of Marmur (1961). DNA–DNA hybridization studies were performed according to the competition procedure of the membrane method of Johnson (1994), described in detail by Arahal *et al.* (2001). The hybridization experiments were carried out under optimal conditions at a temperature of 49.5 °C, which was within the limits of validity for the filter method (De Ley & Tijtgaat, 1970). The percentage of hybridization was calculated as described by Johnson (1994). Three independent determinations were performed for each experiment and the results reported are mean values. The DNA–DNA relatedness between the two representative isolates SN9-4M^T and 466-01 was 74%, demonstrating that they were members of the same species (Wayne *et al.*, 1987). The DNA–DNA reassociation values between strain SN9-4M^T and *M. cuniculi* CCUG 2154^T and *M. pluranimalium* 248-01^T were 17% and 31%, respectively, clearly confirming that the new isolates constituted a separate species (Wayne *et al.*, 1987).

The G+C content of the DNA of strain SN9-4M^T was determined from the mid-point value (T_m) of the thermal

denaturation profile (Marmur & Doty, 1962) obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm. The G+C content of the type strain was 46.6 mol%. This DNA G+C content was within the range reported for members of the genus *Moraxella* (Juni & Bøvre, 2005).

Overall, the results of the present study showed that the new isolates from pigs constituted a distinct branch within the genus *Moraxella* (Fig. 1). Moreover, the new isolates could be distinguished from the nearest phylogenetic relatives, *M. cuniculi* and *M. pluranimalium*, and other phylogenetically closely related species of the genus *Moraxella* on the basis of phenotypic characteristics (Table 1). Given these phenotypic differences and based on phylogenetic and genotypic criteria, it is clear that the new isolates merit classification as a novel species of the genus *Moraxella*, for which the name *Moraxella porci* sp. nov. is proposed. Tests which are useful in differentiating *M. porci* sp. nov. from phylogenetically closely related species of the genus *Moraxella* are shown in Table 1.

Description of *Moraxella porci* sp. nov.

Moraxella porci (por'ci. L. gen. n. *porci* of a pig).

Cells are Gram-negative cocci (1–1.5 µm diameter), non-endospore-forming. Catalase-positive, oxidase-positive and aerobic. Colonies are circular, non-pigmented, smooth, entire and do not produce a diagnostic odour on Columbia blood agar after 48 h incubation at 37 °C. Colonies are non-haemolytic. Growth occurs at 22–42 °C, but not at 15 °C. Growth is enhanced in 5–10 % CO₂ (capnophilically) on 5 % sheep blood agar and chocolate agar. Growth does not occur on MacConkey agar. Growth occurs on brain heart infusion broth containing 0.5–1.5 % (w/v) NaCl, but not with 4 or 6.5 % NaCl. Growth with 3 % NaCl is variable (positive for strain SN9-4M^T). With API 20E and API 20NE kits, aesculin, gelatin and urea are not hydrolysed. Acetoin and indole are not produced (API 20E). Acid is not produced from D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, melibiose, amygdalin or L-arabinose (API 20E). The following carbon substrates are assimilated (Biolog GN Microplates): methyl pyruvate (SN9-4M^T is negative), β-hydroxybutyric acid, α-ketoglutaric acid, D, L lactic acid and succinic acid. Does not assimilate α-D-glucose, citric acid, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, D-gluconic acid, α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, D-arabitol, i-erythritol, D-fructose, D-galactose, myo-inositol, D-mannitol, D-mannose, D-psicose, D-sorbitol, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, bromosuccinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-pyroglutamic acid, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, uridine, 2-aminoethanol, glycerol, DL-α-glycerol phosphate, N-acetyl-D-galactosamine, cellobiose, melibiose, L-fucose, gentiobiose, α-lactose, lactulose, melibiose, methyl β-D-glucoside, raffinose, L-rhamnose, sucrose, D-trehalose, turanose, xylitol, γ-hydroxybutyric acid, itaconic acid, sebacic acid, glucuronamide, glycyl L-aspartic acid, L-phenylalanine, D-serine, thymidine, phenylethylamine, putrescine, 2,3-butanediol, glucose 1-phosphate or glucose 6-phosphate (Biolog GN Microplates), phenylacetate, caprate or adipate (API 20NE). Assimilation of malate, monomethyl succinate and reduction of nitrate are variable (SN9-4M^T is positive). Activity for ester lipase C8, esterase C4, leucine arylamidase and naphthol-AS-BI-phosphohydrolase is detected (API ZYM). Valine arylamidase, lipase C14, alkaline phosphatase, cystine arylamidase, acid phosphatase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, α-glucosidase, N-acetyl-β-glucosaminidase, β-glucosidase, β-glucuronidase, α-galactosidase, β-galactosidase, α-mannosidase,

α-fucosidase, α-chymotrypsin and trypsin are not detected (API ZYM).

The type strain, SN9-4M^T (=CECT 7294^T=CCUG 54912^T), was isolated from the brain of a pig with meningitis. The clinical significance is uncertain. The DNA G+C content of this strain is 46.6 mol% (T_m).

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