

Characterisation of a novel *Mannheimia* sp from Australian feedlot cattle

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Objective To characterise eight isolates of a Gram-negative organism obtained from the upper respiratory tract of cattle showing evidence of mild upper respiratory tract disease.

Design The isolates were compared with the five recognised species within the genus *Mannheimia* - *M haemolytica*, *M glucosida*, *M granulomatis*, *M ruminalis* and *M varigena* - using a range of phenotypic and genotypic methods.

Results Phenotypic characterisation indicated that the isolates belonged to the trehalose-negative [*Pasteurella*] *haemolytica* complex. This complex has recently been reorganised into five species within the new genus *Mannheimia*. Ribotyping performed using *Hind*III and a computerised analysis system indicated that the eight Australian isolates formed a distinct cluster that was related to, but different from, the five recognised species of *Mannheimia*. The 16S rRNA sequence of one isolate (BNO311) was determined and a phylogenetic analysis performed. Isolate BNO311 was distinct from the five named *Mannheimia* spp but did join a larger cluster consisting of rRNA cluster IV (*M varigena*) and the unnamed rRNA cluster V of *Mannheimia*. DNA:DNA hybridisation between isolate BNO311 and *M haemolytica* NCTC 9380^T, *M granulomatis* P411 and *Actinobacillus ligniersii* NCTC 4189^T all suggested similarities of approximately 30%.

Conclusions These phenotypic and genotypic characterisation studies suggest that the eight Australian isolates represent a new species of *Mannheimia*. Until further characterisation studies are performed, we are unwilling to propose a name for this taxon, preferring to refer to this possible new species as Bisgaard taxon 39 of cluster V of *Mannheimia*.

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[*Pasteurella*] *haemolytica* (the brackets indicate that this taxon was excluded from the genus *Pasteurella sensu stricto* in 1985¹) has long been associated with respiratory disease in cattle.² Historically, there have been two biotypes of [*P*] *haemolytica* recognised - biotype A consisting of isolates that ferment L-arabinose and biotype T consisting of isolates that ferment trehalose.^{3,4} A third biotype was proposed for those [*P*] *haemolytica* isolates that did not fit this A/T pattern.⁵

A series of studies suggested that the trehalose-positive isolates within [*P*] *haemolytica* represented a distinct species.⁶⁻⁸ Despite the general acceptance that trehalose-positive [*P*] *haemolytica* are not closely affiliated with the genus *Pasteurella sensu stricto*, these organisms were classified as [*P*] *trehalosi*.⁹ In terms of serovars, [*P*] *trehalosi* contains [*P*] *haemolytica* serovars 3, 4, 10 and 15.⁹

The trehalose-negative [*P*] *haemolytica* complex has recently been the subject of an extensive polyphasic study that has used a range of genotypic and phenotypic methods.¹⁰⁻¹² This has resulted in the proposal of a new genus, *Mannheimia*, for the trehalose-negative isolates of [*P*] *haemolytica*.¹³ In the creation of the genus *Mannheimia*, a total of five new species were recognised - *M haemolytica*, *M glucosida*, *M granulomatis*, *M ruminalis* and *M varigena*.¹³ The bulk of what was once regarded as trehalose-negative [*P*] *haemolytica* is now recognised as *M haemolytica*, specifically [*P*] *haemolytica* serovars 1, 2, 5, 6, 7, 8, 9, 12, 13, 14 and 16. The isolates once recognised as [*P*] *haemolytica* serovar 11 have been reclassified as *M glucosida*.¹³ Serotyping, however, does not result in a clear, species-specific diagnosis.¹⁴

In the current study, we describe the isolation and characterisation of a collection of *Mannheimia*-like organisms from Australian feedlot cattle.

Materials and methods

Isolates

All eight Australian isolates examined in this study were isolated from nasal swabs obtained from cattle in a feedlot. The nasal swabs were cultured by inoculation onto 5% sheep blood agar. Three sequential single colony picks were performed to ensure each isolate was pure. The organisms were then stored by freeze-drying. A fresh ampoule was opened each time for characterisation work. The reference strains used in this study (listed in Table 1) are all held in the collection of Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark.

Phenotypic characterisation

A full phenotypic characterisation was performed using methods previously described.¹⁵

Ribotyping

Subcultures from stocks kept at -80°C were made on blood agar plates (Oxoid Blood Agar Base III supplemented with 5% bovine blood), which were incubated for 24 h at 37°C. Single colonies were transferred to Luria-Bertani broth¹⁶ supplemented with 5% bovine serum. The cultures were grown overnight with shaking at 37°C. Isolation of total DNA, restriction digestion using the enzyme *Hind*III (Boehringer GmbH, Mannheim, Germany), separation on 0.8% agarose gels, DNA blotting, and hybridization at 56°C with a digoxigenin-labelled probe (Boehringer) complementary to 16S

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Table 1. Properties of Australian isolates of *Mannheimia* sp separating this taxon from the type or reference strains of the recognised *Mannheimia* species.^a

	Australian strains (Taxon 39)	<i>M haemolytica</i> NCTC 9380 ^T	<i>M glucosida</i> CCUG 38457 ^T	<i>M ruminalis</i> CCUG 38470 ^T	<i>M granulomatis</i> ATCC 49244 ^T	<i>M varigena</i> CCUG 38462 ^T	<i>Mannheimia</i> sp R 19.2	<i>Mannheimia</i> sp HPA 121
Oxidase	-	+	+	+	W	+	+	+
β-haemolysis	W	W	W	-	-	W	W	-
ODC	-	-	+	-	-	+	-	-
Indole	-	-	-	-	-	-	(+)	+
Acid from								
D(-) arabinose	-	-	W	(+)	W	W	-	-
L(+) arabinose	-	-	-	-	-	+	+	+
Dextrin	+(+)	+	+	-	(+)	W	(+)	(+)
L(-) fucos	-	+	+	-	W	+	-	W
D(+) galactose	-	+	+	+	(+)	+	+	+
Glycerol	-	-	(+)	-	(+)	(+)	(+)	-
Meso-inositol	-	W	(+)	-	W	(+)	+	(+)
Maltose	+(+)	+	+	-	(+)	+	+	+
Raffinose	D	(+)	(+)	-	-	(+)	(+)	(+)
L(+) rhamnose	-	-	-	-	-	+	-	-
D(-) sorbitol	D	+	+	-	+	-	-	+
D(+) xylose	-	+	+	-	-	+	+	+
ONPG	-	-	+	+	+	+	+	+
Glycosides ^b	-	-	+	-	+	-	-	-
α-fucosidase	D	+	+	-	-	+	-	W
β-glucuronidase	-	+	-	-	+	-	-	-
β-xylosidase	-	-	+	-	-	-	+	-
No. of differences with Australian strains		5	10	6	5	10	9	7

^a ODC = ornithine decarboxylase; W = weak positive; + = positive within 1-2 days, (+) = delayed positive within 14 days; D = variable reaction, - = negative after 14 days

^b Glycosides are aesculin, amygdalin, arbutin, cellobiose, gentiobiose and salicin

and 23S rRNA of *Escherichia coli* (Sigma) were performed as previously described.¹²

Analysis of ribotype patterns

The ribotype patterns were analysed using GelCompar (Applied Maths BVAB). A mixture of digoxigenin labelled molecular markers II and III (Boehringer) was used in every 5th lane for standardisation of gels. The band positions were analysed using fine optimisation and 0.8% position tolerance. The similarity between individual strains and the type strains of the five *Mannheimia* species and the three strains representing the unnamed rRNA cluster V of *Mannheimia*¹³ was estimated using the Dice coefficient as a measure of similarity. Cluster analysis based on the similarity matrix obtained was done using the Unweighted Pair Group Method of Arithmetic Averages (UPGMA).

Amplification and sequencing of 16S rRNA

The small subunit (16S) rRNA gene was amplified directly from cell biomass using 27f and 1525r primers.¹⁷ A 1-μL sterile disposable loop was used to lift a small amount of colony material from the surface of an overnight blood agar plate into 200 μL sterile phosphate buffered saline in a 0.6-mL tube. After vortexing, the suspension was heated on the thermal cycler at 98°C for 15 min. The cell debris was pelleted by centrifuging at maximum speed in a benchtop microfuge for 5 min and 1 μL of the supernatant was used as template. The 50-μL reaction mixture contained 1 mM MgCl₂, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 100 μg/mL gelatin, 200 mM each dNTP (dATP,

dCTP, dGTP, dTTP), 0.4 μM of each primer 27f and 1525r, 1 μL of template and 0.8 units *Taq* DNA polymerase (Boehringer Mannheim). The PCR was performed using a Hybaid OmniGene Thermal Cycler (Hybaid, Middlesex, UK). An initial heating step at 98°C for 2.5 min was performed prior to the addition of the *Taq* DNA polymerase. A 50-μL mineral oil overlay was then added. The following cycling parameters were used: denaturing at 93°C for 1 min, annealing at 52°C for 45 s and extension at 72°C for 2 min, these three steps being repeated for 30 cycles. This was followed by a final cycle with an extension step of 10 min. The PCR product for sequencing was purified using the QIAquick PCR Purification kit (QIAGEN GmbH, Hilden, Germany). DNA sequencing was performed using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase FS and a model 373A DNA sequencing system (PE Applied Biosystems Inc., Foster City, CA, USA). All procedures were performed according to the manufacturer's protocols. Both strands of the 16S rDNA were sequenced using the primers 27f, 342r, 357f, 519r, 530f, 787r, 803f, 1100r, 1114f, 1241f, 1392r and 1492r.¹⁸ The sequence determined for BNO 311 was deposited in EMBL under accession number AF216870.

Phylogenetic analysis of sequence data

The sequence of strain BNO311 was aligned with selected sequences from GenBank and analysed using the ARB program.¹⁹ Maximum likelihood analysis was performed using fastDNAm1.²⁰ Bootstrapping (2000 resamplings) was performed according to previously described methods.²¹

DNA:DNA hybridisation

Cells were grown under shaking at 37°C in Tryptic Soy Broth (Difco) supplemented with 0.4% filter sterilized yeast extract (Oxoid) and 0.01 M NaHCO₃. The cells were harvested in the early-stationary phase of growth, examined microscopically for purity, washed in 1 X SSC (0.15 M NaCl, 0.015 M trisodium citrate) and resuspended in 1 X SSC containing 0.01 M EDTA. High-molecular-mass DNA was isolated as previously described.¹³ Initial renaturation rates of homologous or heterologous solutions of DNA were determined by a previously described method²² using a model 250 spectrophotometer (Gilford Instruments). A DNA concentration of 80 µg/mL, a salt milieu containing 2 X SSC and a reassociation temperature of 68°C were used. The reassociation temperature was calculated as previously proposed.²³ The DNA binding values (D%) were calculated according to a published formula.²² Each experiment was repeated at least three times.

Results

Isolation

Each isolate was obtained from a different animal within the one herd, with all animals suffering from mild respiratory disease. Although no other bacterial pathogen was isolated, all eight isolates were obtained as part of a mixed flora. The isolates were recognised due to the weak zone of haemolysis surrounding the colonies.

Phenotypic characterisation

All eight field isolates were Gram-negative, non-motile rods that fermented glucose without the production of gas, did not produce urease or indole and lacked oxidase activity. The isolates all showed weak haemolysis of both bovine and ovine blood cells. The isolates had no requirement for X- or V-factors in vitro, were unable to utilise citrate, mucate or malonate, did not produce H₂S, did not produce any pigment, could not grow in potassium cyanide and were negative in the methyl red and Vogues-Proskaur tests. All the isolates could reduce nitrate without the formation of gas but lacked the ability to hydrolyse arginine and decarboxylate lysine or ornithine and could not hydrolyse gelatin, Tween 20 or Tween 80. None of the isolates could grow on MacConkey agar or deaminate phenylalanine but all showed catalase and phosphatase activity. All the isolates were negative for α-galactosidase, α-glucosidase, α-mannosidase, β-galactosidase, β-glucosidase, β-glucuronidase and β-xylosidase activity but all were positive for alanine aminopeptidase activity. None of the isolates produced acid from adonitol, aesculin, amygdalin, D(-) arabinose, L(+) arabinose, D(+) arbutin, arbutin, cellobiose, dulcitol, meso-erythritol, D(+) fucose, L(-) fucose, D(+) galactose, gentiobiose, β-N-CH₃-glucosamide, glycerol, D(+) glycogen, meso-inositol, inulin, lactose, D(+) mannose, D(+) melibiose, D(+) melezitose, L(+) rhamnose, salicin, L(-) sorbose, trehalose, D(+) turanose, xylitol, D(+) xylose or L(-) xylose. All the isolates produced acid from dextrin, D(-) fructose, D(+) glucose, D(-) mannitol, maltose, D(-) ribose and sucrose. The isolates differed in the ability to produce acid from raffinose and D(-) sorbitol with only two and three isolates, respectively, being positive for these properties. Five of the eight isolates were positive for α-fucosidase activity.

The above properties indicated that the isolates were in the trehalose-negative [*P*] *haemolytica* complex. However, comparison of the properties of the field isolates with the type or reference strains of the recognised *Mannheimia* species (see Table

1), showed that the Australian field isolates were clearly distinct. The nearest matches in phenotypic properties were *M haemolytica* and *M granulomatis* and even then, there were five differences with both species.

Ribotyping

The eight Australian field isolates all yielded unique but similar ribotype patterns. The patterns contained between 14 to 23 well-resolved bands. Estimates of the degree of similarity between the field isolates and the type or reference strains for the recognised *Mannheimia* spp are presented in the phenogram shown in Figure 1. Seven of the Australian field isolates formed a cluster at a similarity of 78%. The eighth Australian isolate joined this cluster at a similarity of 72%. This Australian cluster did not join with the rest of the *Mannheimia* strains until a similarity of less than 30% was reached. These clustering results indicate that the Australian isolates represent a coherent cluster, which is clearly distinguishable from the recognised *Mannheimia* species.

Phylogenetic analysis

Phylogenetic analysis of the 16S rRNA gene sequence of strain BNO311 showed that the isolate could not be reliably assigned to any of the five currently recognised 16S rRNA clusters.¹³ A phylogenetic tree showing the relationship of strain BNO311 in relation to the genus *Mannheimia* and selected reference strains in the family *Pasteurellaceae* is shown in Figure 2. The bootstrapping analysis showed that strain BNO311 is not reliably linked with the other members of the genus *Mannheimia* until rRNA clusters IV (*M varigena*) and V (a collection of un-named *Mannheimia* isolates) merge. As expected, the bootstrapping analysis supported the other previously recognised clusters – I *M haemolytica/M glucosida*, II – *M ruminalis*, III – *M granulomatis* and IV – *M varigena*.

DNA:DNA hybridisation

The DNA:DNA hybridisation work with strain BNO311 and selected type/reference strains resulted in DNA relatedness values of 33% ± 7% (*M haemolytica* NCTC 9380^T), 30% ± 3% (*M granulomatis* P 411) and 28% ± 7% (*Actinobacillus lignieresii* NCTC 4189^T).

Discussion

The phenotypic characterisation of the eight Australian isolates indicated that they were members of the trehalose-negative [*P*] *haemolytica* complex, which has recently been extensively re-organised and placed within the new genus *Mannheimia*.¹³ However, the isolates did not match any of the five recognised species nor representatives of the currently un-named taxa within the genus *Mannheimia* (see Table 1).

Ribotyping was originally proposed as a taxonomic tool by Grimont and Grimont.²⁴ It has been suggested that the value of ribotyping for the calculation of taxonomic distances between strains is questionable.²⁵ The caution on the use of ribotyping arises from the fact that ribotype fragments of similar size may not represent homologous DNA sequences and that the fragments are not independent as removal of a single restriction site may result in a difference of three bands.¹³ Nevertheless, ribotyping has proven a useful taxonomic tool in diverse bacterial groups including the family *Pasteurellaceae*. Ribotype clusters have been shown to be a useful way of separating *Actinobacillus equuli* isolates and related species.²⁶ Ribotyping

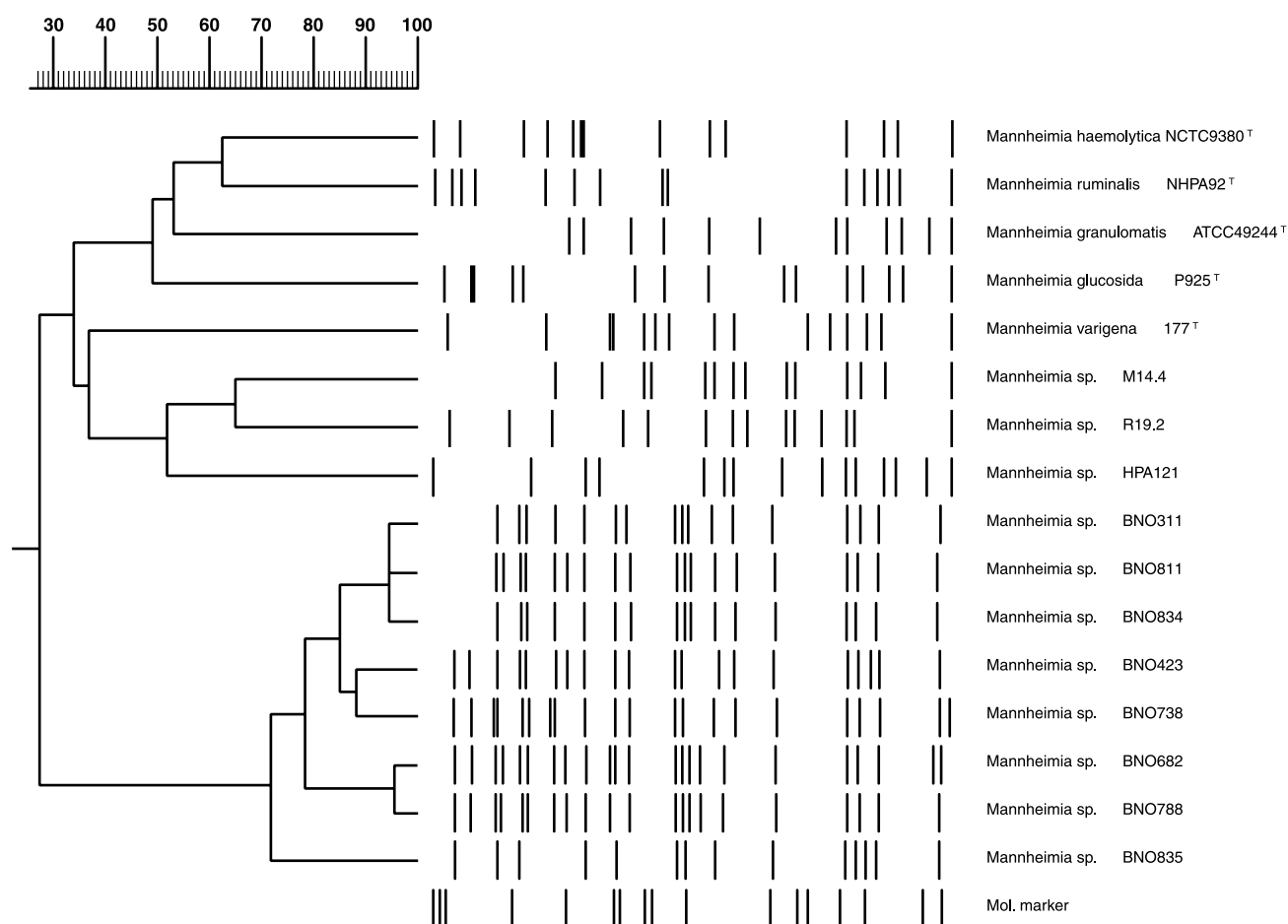


Figure 1. Analysis of ribotype patterns of eight Australian isolates and selected type and reference strains from the genus *Mannheimia* based on *Hind*III digestion, Dice coefficient of similarity and clustering by UPGMA. Fragment sizes of molecular weight marker (bp): 27491, 23130, 21226, 9416, 6557, 5148, 4973, 4361, 4268, 3530, 2322, 2027, 1904, 1584, 1357, 947 and 831.

clusters of both avian *Pasteurella multocida* and trehalose negative [*P*] *haemolytica* have been shown to correlate with multi-locus enzyme electrophoresis (MLEE) clusters.^{12,27} As MLEE clusters have been shown to correlate with measures of overall DNA homology as revealed by DNA:DNA hybridisation in a range of bacteria,²⁸⁻³¹ this is further evidence of the applicability of ribotyping.

Ribotyping clearly showed that the eight Australian isolates were a distinct cluster that did not join the recognised *Mannheimia* spp until a very low-level similarity of less than 30% (see Figure 1). Furthermore, the ribotyping results indicated that the Australian isolates were a tight cluster with all eight isolates sharing at least 72% similarity. The figure of 70% similarity for ribotype clusters within the trehalose-negative [*P*] *haemolytica* complex has been shown previously to result in clusters that match with the new species within the genus *Mannheimia*.¹³ Hence, the results of our ribotyping work suggest that the eight Australian isolates represent a new species within the genus *Mannheimia*.

Analysis of the 16S rRNA sequence of Australian isolate BNO311 (see Figure 2) established that the isolate could not be reliably linked with any of the five currently recognised rRNA clusters described by Angen et al.¹³ Isolate BNO311 is reliably

linked with the larger cluster formed by rRNA Clusters IV (that is, *M varigena*) and V (a collection of un-named isolates). Bovine isolates of *M varigena* have been associated with disease conditions such as pneumonia, mastitis and septicaemia as well as being isolated from non-sterile sites such as the rumen, oral cavity and intestine.¹³ The Cluster V isolates have been obtained from bovine rumen (HPA121, R19.2) or a bovine abortion (PH704).¹³ The distinctive nature of isolate BNO311 was further confirmed by the DNA:DNA hybridisation results in which we found no relatedness value of greater than 33% with representatives of *M haemolytica*, *M granulomatis* and *A lignieresii*.

Although the isolates were all obtained from cattle with respiratory disease, this is not convincing evidence that the Australian isolates are linked to bovine respiratory disease. The isolates were obtained in mixed growth from the upper respiratory tract. It is possible that the isolates may be a component of the normal microbiota and that similar organisms can be found in the upper respiratory tract of healthy cattle. We hope that this description of the phenotypic and genotypic properties of the Australian isolates will result in other laboratories developing the capacity to identify these organisms. With a range of laboratories capable of recognising these

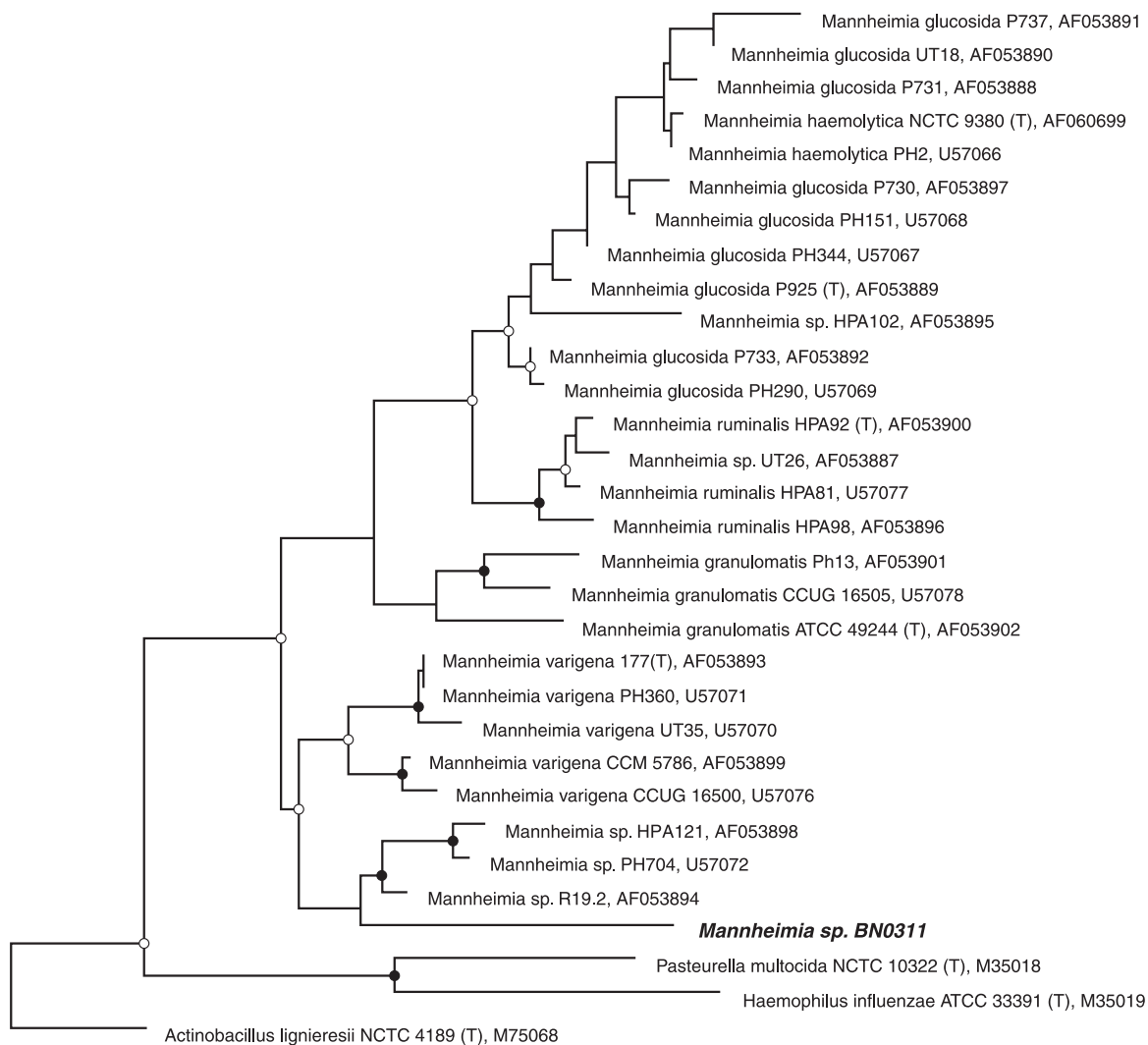


Figure 2. Phylogenetic position of BNO311 in relation to genus *Mannheimia* and selected type strains representing other genera of the family *Pasteurellaceae*. Branch points supported (bootstrap values, >75%) by all inference methods used are indicated by solid circles, and those supported by most inference methods are indicated by open circles. Branch points without circles were not resolved (bootstrap values, <75%).

organisms, it should be possible to develop a clearer picture of the role of these organisms in bovine respiratory disease.

Overall, the phenotypic and genotypic results indicate that the Australian isolates appear to represent a new taxon within the genus *Mannheimia*. However, further DNA:DNA hybridisation studies are necessary to support the formal proposal of a new species. Hence, for the moment, we suggest the use of Bisgaard Taxon 39 of *Mannheimia* rRNA cluster V to refer to these organisms.

Acknowledgments

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BOOK REVIEW

Social Behaviour in Farm Animals. Edited by Keeling LJ and Gonyou HW, CABI Publishing, Wallingford, 2001, 432 pages. Price US\$ 110.00. ISBN 085199 397 4.

This book is a welcome addition to the excellent series of animal behaviour and welfare texts produced by CABI over the last 10 years. The book's objective is to describe the social behaviour of farm animals and how this is affected in modern management systems.

The book is divided into three sections; Concepts in Social Behaviour (4 chapters), The Social Behaviour of Domestic Animals (6 chapters) and Contemporary Topics in Social Behaviour (4 chapters). The first section deals with the evolution of social behaviour including domestication and has a chapter on parental behaviour. The second section is descriptive with a chapter on the social behaviour of each of the common farm species, cattle, pigs, domestic birds, sheep, horses, and fish. The final section looks at the role of humans in the life of farm animals, the effects of breaking social bonds, animal personalities and social cognition.

Each chapter is written by one or more recognised authorities in the subject area. It is pleasing to note that the chapter on sheep was written by Andrew Fisher and Lindsay Matthews from New Zealand. The editors preface each chapter with a brief comment on its significance. Each chapter is a review of the subject and many identify areas still to be investigated.

The social behaviour of water buffalo, goats, camels, deer and dogs are not described. The species covered are those that have had the most research carried out on them over the last half century. Much of that research was stimulated by concern about animal welfare in intensive farm management systems.

This is an excellent book. It is easy to read, thorough and interesting. If you are interested in farm animals and in how they live you will enjoy this book. The evolutionary perspective is unusual in animal science textbooks because of concerns about its relevance to domesticated animals. This is recognised by the authors but they consider it to be a valuable background for the understanding of social behaviour.

The chapters on the social behaviour of farm animals, poultry and fish are all of a high standard and should be of value to veterinarians with an interest in any of these species. Those with an interest in a particular species will be familiar with much of the material but reading an up-to-date review is worthwhile and may boost one's ego a little.

The section on humans and farm animals does not feature Paul Hemsworth and this is unusual given his expertise in the area. This section contains an interesting discussion on humans in the world of animals by Jeffrey Rushen and others.

This is a book to read for pleasure and information. In a world where veterinarians are expected to know not only about health and productivity but also about 'welfare' this book is very useful. It is of particular interest to everyone interested in farm animal behaviour and welfare. It is recommended Christmas reading for those who cannot be bothered to read traditional holiday blockbusters.

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