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Mycoplasma capricolum subsp. *capripneumoniae*, the cause of contagious caprine pleuropneumonia, comprises two distinct biochemical groups

Zeina Soayfane[1], Bilal Houshaymi[1] and Robin A.J. Nicholas[2,*]

[1]*Microbiology Department, Lebanese University, Hadath Campus, Beirut, Lebanon*
[2]*Consultant, The Oaks, Nutshell Lane, Upper Hale, Farnham, Surrey GU9 0HG, UK*

Abstract

Mycoplasma capricolum subsp. *capripneumoniae*, the cause of the World Organisation of Animal Health- listed contagious caprine pleuropneumonia, is a member of the *Mycoplasma mycoides* cluster which comprises five pathogenic mycoplasmas of ruminants. These mycoplasmas are closely related immunologically and genetically which can lead to difficulties for differential diagnosis. The patterns of substrate metabolism of strains of *M. c. capripneumoniae*, gathered from diverse geographic regions, were studied by measurement of oxygen uptake rates. The strains fell into two major biochemical groups: one which only oxidised organic acids and glycerol and the other which could additionally metabolise sugars. Furthermore when DNA-DNA hybridisation tests were carried out these two groups of strains could be separated by their degree of DNA homology, the mean hybridisation value between members of the two groups was 86% well above the value of 70% normally used to indicate separate species. DNA-DNA hybridisation was also carried out between *M. c. capripneumoniae* strains and other members of the *M. mycoides* cluster. These experiments used labelled DNA from two representative subsp. *capripneumoniae* strains; these were 7/1a (organic acid-oxidising) and 4/2 LC (glucose-oxidising). The results showed a particularly close relationship of the glucose-oxidising strain to *M. leachii* strains.

Keywords: *Mycoplasma capricolum* subsp. *capripneumoniae*, Contagious caprine pleuropneumonia, Substrate metabolism, DNA-DNA hybridisation.

Introduction

Mycoplasma capricolum subspecies *capripneumoniae* is a member of *Mycoplasma mycoides* cluster (Cottew *et al*., 1987) which comprises five pathogens of ruminants including the mycoplasmas causing contagious bovine pleuropneumonia, contagious caprine pleuropneumonia (CCPP) and contagious agalactia which have been listed by the World Organisation for Animal Health because of their damaging socio-economic impacts. CCPP is characterised by respiratory distress, coughing, nasal discharge and severe fibrinous pleuropneumonia. It is a significant disease of goats in Africa, the Middle East and parts of Asia, and can cause mortality of up to 80% in immunologically naïve herds (Houshaymi *et al*., 2002). The members of the *M. mycoides* cluster show close genetic and immunological similarity which has led to difficulties for differential diagnosis (Leach *et al*., 1993).

A study of their substrate metabolism was first carried out by Abu-Groun *et al*. (1994) who showed that it was possible to differentiate and classify groups within the cluster by their patterns and kinetics of substrate utilisation. In that study, only one of four strains of *M. c. capripneumoniae* oxidised glucose; the other three gave no detectable oxygen uptake with glucose but did oxidise organic acids. These results were surprising since all strains of the very closely related *M. c. capricolum* and other members of the cluster tested oxidised glucose. However, for some of the cluster, *M. mycoides* subsp. *mycoides (*previously suffixed SC), *M. leachii* (previously named bovine serogroup 7) and *M. c. capripneumoniae*, only small numbers of strains were tested.

Pettersson *et al*. (1998) was the first to study the polymorphism patterns amongst 16S rRNA genes of *M. c. capripneumoniae* from diverse locations. While it appeared that the mycoplasma formed a homogenous group, a phylogenetic tree constructed by comparative analysis of the polymorphisms, revealed two distinct lines of descent. Other studies on evolutionary and genetic relationship between the members of the '*M. mycoides* cluster' have been published over the last decade increasing our understanding of the molecular biology of these mycoplasmas (Manso-Silvan *et al*., 2007; Thiaucourt *et al*., 2011), less is known of the biochemistry of these mycoplasmas, in particular the patterns and kinetics of substrate utilisation.

In the work reported here, additional strains were included to investigate further the diversity of substrate utilisation with specific reference to *M. c. capripneumoniae*.

***Corresponding Author:** Dr. Robin A.J. Nicholas. Consultant, The Oaks, Nutshell Lane, Upper Hale, Farnham, Surrey GU9 0HG, UK. *Tel*.: +44 1252725557. Email: *robin.a.j.nicholas@gmail.com*

In addition, DNA-DNA hybridisation was used to investigate the relationships between the strains and other members of the cluster. The aims of the work were to determine the genetic homogeneity amongst strains which might enable the subdivision of strains in accordance with the biochemical findings.

Materials and Methods

Mycoplasma strains

M. c. capripneumoniae strains used in this work were 19/2, 4/2LC (Oman), T3, T7, T9, T11 (Eritrea), Baringo, F38, G94/83 (Kenya) and 7/1a (Turkey). They were obtained from the strain bank of the Veterinary Laboratories Agency (now Animal and Plant Health Agency), Addlestone, UK. Their identity was confirmed by serological and molecular amplification methods (Houshaymi *et al*., 2002). The Mycoplasma bovis type strain NCTC 10131was included as a negative control in one hybridisation experiment.

Biochemical studies

Substrate oxidation was based on measurement of oxygen uptake, detected by changes in dissolved oxygen tension (DOT) using an oxygen electrode system as described by Miles and Agbanyim (1998). The sugars and organic acids tested as possible metabolic substrates included all those substrates previously shown to be oxidised by members of the *M. mycoides* cluster in the extensive study of Abu-Groun *et al*. (1994).

Labelling of mycoplasma DNA with digoxigenin

A small amount (1 µg) of the reference DNA, extracted by the method of Bashiruddin (1998), to be labelled was digested with *Eco*RI enzyme at 37 °C for 4 to 16 h. The standard volume of the digestion mix was 20 µl. After digestion this was adjusted to 100 µl with deionised water and the digest was then extracted with an equal volume of phenol/chloroform (50:50). The DNA in the aqueous phase was then precipitated with 2 volumes of cold ethanol and collected by centrifugation at 12,000 rpm for 20 min. The DNA pellet was air dried for 30 min and then dissolved in 15 µl of TE buffer. Prior to labelling, the DNA was denatured by heating in a boiling water bath for 5 min and then cooling rapidly on ice.

The denatured DNA was labelled overnight at 37 °C using 2 µl hexanucleotide mixture, 2 µl digoxygenin-labelled dNTP and 1 µl Klenow enzyme (Roche,Welwyn Garden City, UK).

The labelling reaction was stopped by adding 2 µl of 0.2 M EDTA. The labelled DNA was then precipitated by adding 2.5 µl of 4 M LiCl and 75 µl of cold ethanol. The mixture was maintained at –20 °C for 2 h and the precipitate was then collected as before and air-dried for 30 min. The DNA was re-dissolved in 50 µl of TE buffer.

Preparation of DNA-loaded membranes for hybridisation

Target DNA to be analysed was blotted onto Hybond N^+ charged nylon membranes (Amersham International, Amersham, UK), using a standard vacuum dot blot apparatus. The wells of the apparatus were loaded with 3 µg of purified genomic DNA (contained in approximately 10 µl TE buffer). The membrane was applied for 5 min to filter paper wetted with denaturation buffer (1.5 M NaCl and 0.5 M NaOH) and then to filter paper wetted with neutralisation buffer (0.5 M Tris-HCl, pH 8.0, 3 M NaCl) for a further 5 min. Finally, the membrane was exposed (after air drying) to a UV trans-illuminator (312 nm) for 4 min to immobilise the DNA.

DNA hybridisation

Hybond N^+ membranes loaded with target DNA were hybridized overnight with 100 ng of freshly denatured, digoxigenin-labelled reference DNA at 68 °C. The protocol adopted was as described for the DIG Labelling and Detection Kit by the manufacturer (Roche, Welwyn Garden City, UK). The hybridisation buffer (25 ml) was 5 x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, and 1% (w/v) blocking reagent. Blocking reagent was prepared as a stock of 10% (w/v) solution of this reagent (Roche, Welwyn Garden City, UK) dissolved in buffer A (0.1 M maleic acid and 0.015 M NaCl, pH 7.0) by constantly stirring on a heating block (65 °C) for 1 h and then autoclaving and storing at 4 °C. SSC was prepared as a stock solution of 20x SSC (3 M NaCl and 0.3 M sodium citrate, pH 7.0).

Membranes were washed twice with 50 ml of washing buffer I (2 x SSC containing 0.1% (w/v) SDS) at room temperature for 5 min and then twice (15 to 30 min each) in washing buffer II (0.1x SSC and 0.1% (w/v) SDS) at 68 °C. After hybridisation, the membranes were washed for 30 min at room temperature with 100 ml of buffer A and then for a further 30 min with 100 ml of blocking reagent diluted 1:10 in buffer A. Anti-digoxigenin antibody (Roche,Welwyn Garden City, UK) was then diluted 1:5000 in 20 ml of the same blocking reagent solution and applied to the membrane for 30 min.

After incubation with antibody the membrane was washed twice in buffer A (100 ml) for 15 min at room temperature and then twice in 0.5 x SSC (100 ml) for 10 min at 65 °C. The membranes were then immersed in 20 ml of buffer B (0.1 M Tris-HCl, 0.1 M NaCl and 0.05 M $MgCl_2$, pH 9.5) for 2 min. Finally, the membranes were incubated in a freshly prepared colour-substrate solution (200 µl of a NBT/BCIP stock solution in 10 ml of buffer B), sealed in a plastic box in the dark and incubated for 16 h at room temperature. The reaction was stopped by washing the membranes

for 5 min with 50 ml of water. Membranes were then analysed with an image analyser, using a programme written by Dr L. Richards, King's College London.

Results

Biochemical studies

Rates of oxygen uptake with glucose, organic acids, glycerol and ethanol are compared in Table 1 which are expressed as a percentage of the rate observed with lactate (50 µM) rather than with glucose. The reason for this was that glucose was not metabolised by all strains. Amongst the eight new strains, strains 19/2 and 4/2 LC and Eritrean strains T3, T7, T9 and T11 were able to metabolise glucose (Table 1).

In contrast, Baringo and 7/1a, were unable to utilise glucose, even at a high concentration of 2.5 mM but rapidly metabolised the organic acids, lactate, pyruvate and 2-oxobutyrate. The ability of strain F38 to oxidise organic acids, but not glucose, was shown. The specific rates of lactate oxidation for the strains studied varied approximately ten-fold with the highest rates being observed for the Eritrean strains (Table 2).

The six glucose oxidising strains were metabolically uniform in that they all: oxidised N-acetylglucosamine, pyruvate and lactate with a high affinity; oxidised mannose, glucosamine and 2-oxybutyrate with a low affinity; and were unable to utilise maltose, trehalose, fructose or ethanol (Table 3).

Also, five of these six strains oxidised glycerol at a high rate and with a high affinity, the exception being strain 19/2 which was unable to oxidise this substrate.

The subsp. *capripneumoniae* strains unable to oxidise glucose, Baringo, 7/1a, both showed a high affinity for lactate, 2-oxybutyrate and pyruvate (Table 4).

They appeared metabolically similar to the strains, F38, G1943/80, G94/80, studied by Abu-Groun *et al*. (1994) except with respect to glycerol and ethanol oxidation. The strains studied here oxidised glycerol but not ethanol, whereas the converse was true for strains F38, G1943/80 and G94/80.

Molecular studies

The results given in Table 5 show DNA-DNA hybridisation values amongst two glucose-oxidising, 4/2 LC, 19/2, and three organic acid-oxidising, F38, 7/1a, Baringo, strains. Irrespective of the source of the labelled DNA, hybridisation values were mostly greater for strains belonging to the same biochemical group. The mean hybridisation value within the biochemical groups was 94%, whereas between groups it was 85.5%.

This data therefore suggests that the loss of glucose oxidising ability is associated with a particular evolutionary line within subsp. *capripneumoniae*. This is also supported by the data of Pettersson *et al*. (1998) which revealed two major lines of descent amongst subsp. *capripneumoniae* strains (giving polymorphism patterns I and II). The two glucose oxidising strains used in the study were both typed as possessing a IIB3 polymorphism pattern (Table 6).

The organic acid-oxidising strains typed as polymorphism pattern 1A, IIB or IIB2. By DNA-DNA hybridisation, the Baringo strain (pattern IA) appeared more closely related to strain 7/1a (pattern IIB2) than to F38 (pattern IIB).

DNA-DNA hybridisation was also carried out between subsp. *capripneumoniae* strains and other members of the *M. mycoides* cluster (Table 7).

These experiments used labelled DNA from two representative subsp. *capripneumoniae* strains; these were 7/1a (organic acid-oxidising) and 4/2 LC (glucose-oxidising). The results showed a particularly close relationship of the glucose-oxidising strain to *M leachii* strains, with hybridisation values of 88 - 98% (mean 94%; seven strains tested). This relationship was also noted in experiments using labelled DNA from strain B144 P. It is known that *M. c. capripneumoniae* may show strong serological cross reactions with *M leachii* strains (Kanyi Kibe *et al*., 1985; Chingi *et al*., 1987). For the organic acid oxidising strain 7/1a, hybridisation values with *M leachii* strains were lower (77 - 84%) and were similar to those with *M. mycoides* subsp. *mycoides* strains (76 and 83%).

Discussion

In the study of Abu-Groun *et al*. (1994), only four strains of *M. c. capripneumoniae* were used. One of these oxidised glucose while the remaining three strains (including the type-strain, F38) gave no detectable oxygen uptake with glucose but did oxidise organic acids. These results were surprising since the seven *M. c. capricolum* strains and all of the other *M. mycoides* cluster strains in the study oxidised glucose. Also, the metabolic diversity within *M. c. capricolum* and particularly within subsp. *capripneumoniae* was greater than would be expected in a homogeneous taxonomic grouping. Strains unable to utilise glucose presumably differ from glucose-metabolising strains in the possession and/or expression of a wide range of transport proteins and enzymes associated with sugar metabolism and the Embden-Meyerhoff pathway

The data presented in this paper confirm that reported by Abu-Groun *et al*. (1994). Of the eight new strains studied, six metabolised glucose and two did not. In addition, it was confirmed that the type strain of *M. c. capripneumoniae*, F38, also failed to metabolise glucose.

The patterns of substrate utilisation shown by the glucose-metabolising strains were generally similar to one another and to the previously studied strain G183. Amongst the *M. mycoides* cluster, the patterns were most similar to those for *M. m. mycoides* strains; however, all of the glucose-oxidising subsp. *capripneumoniae* strains failed to oxidise fructose and had a low affinity for 2-oxybutyrate.

Table 1. Utilisation of glucose, glycerol and ethanol by *M. c. capripneumoniae* strains.

Substrate	Concentration (µM)	Rate of oxygen uptake (% rate for lactate)										
		19/2	4/2 LC	T3	T7	T9	T11	G183	Baringo	7/1a	F38	G94/83
Lactate	50	100	100	100	100	100	100	100	100	100	100	100
Pyruvate	50	30	22	26	16	15	30	19	75	147	71	217
2-oxybutyrate	40	<1*	<1	<1	<1	<1	<1	<1	69	116	58	191
	400	<1	<1	7	2	3	5.5	<1				
	4.0 mM	18	16	17	22	25						
Glucose	25	44	65	39	52	48	23	590	<1	<1	<1	<1
	2.5 mM								<1	<1	<1	<1
Glycerol	50	<1	72	77	13	79	57	650	85	59	<1	<1
	5.0 mM	<1									<1	<1
Ethanol	10 mM	<1	<1	<1	<1	<1	<1	<1	<1	<1	37**	37

(*): <1 not detectable (<1 nmol^{-1} mg cell protein^{-1}); (**): Data from Abu-Groun *et al.* (1994).

Table 2. Specific rate of lactate oxidation by *M. c. capripneumoniae* strains (Initial lactate concentration was 50 µM).

Strain	Origin	Rate of oxygen uptake with lactate (nmol.min^{-1} cell protein $^{-1}$)
19/2	Oman	32
4/2LC	Oman	29
T3	Eritrea	289
T7	Eritrea	128
T9	Eritrea	171
T11	Eritrea	160
7/1a	Turkey	41
F38	Kenya	37
Baringo	Kenya	45

Table 3. Utilisation of glucose, glycerol and ethanol by *M c.capripneumoniae* strains.

Substrate	Conc. (µM)	Rate of oxygen uptake (% rate for lactate)					
		19/2	4/2 LC	T3	T7	T9	T11
Lactate	50	100	100	100	100	100	100
Maltose	1 nmM	<1*	<1	<1	<1	<1	<1
Trehalose	1 mM	<1	<1	<1	<1	<1	<1
Fructose	25	<1	<1	<1	<1	<1	<1
GlcNAc**	25	19	32	9	28	30	6
Glucosamine	25	9	15	7	14	8	4
	250	31	56	15	25	NT	NT
	2.5 mM	58	100	33	43	NT	NT
Mannose	25	<1	<1	9	5	16	3
	250	5	23	19	11	NT	NT
	2.5 mM	34	41	41	25	NT	NT

(*): <1 not detectable (<1 nmol^{-1} mg cell protein^{-1}); (**): N-acetylglucosamine; (NT): not tested.

The non-glucose oxidising subsp. *capripneumoniae* strains, from both this study and the study of Abu-Groun *et al*. (1994), were also very similar to one another. Interestingly, they all had a high affinity for 2-oxybutyrate. These strains would appear dependent entirely upon organic acids (and possibly glycerol in some strains) for energy generation, and the yield of these strains was increased markedly by pyruvate (data not shown; Miles *et al*., 1988). It was noted that the Eritrean strains (glucose oxidising) were difficult to grow and that their growth yield in media containing glucose was increased several fold by the addition of pyruvate. Thus, it may be that organic acids are also important energy sources for glucose oxidising strains and that the glucose-catabolising pathway in these strains is not used *in vivo* for energy generation. All of the glucose oxidising strains (except G183) metabolised organic acids at significantly higher rates than glucose.

The Eritrean strains used in the present study were isolated from outbreaks in goat herds with very high mortality rates (Houshaymi *et al*., 2002).

All strains metabolised glycerol at a high rate, a feature potentially important in pathogenicity because of the accompanying hydrogen peroxide production which may have accounted for the seriousness of the outbreaks.

However, amongst other isolates, glycerol metabolism varied. Incidently, the high levels of hydrogen production produced by the subsp. *capripneumoniae* strains, in particular those from Eritrea, compared to other members of the cluster (i.e. typically 1 mol compared to about 0.05 mol per mol of O_2 taken up) may account for their fastidiousness in vitro.

The use of pyruvate in the medium may reduce the impact of H_2O_2 production as it may be metabolised to lactate plus acetate without net NADH production.

Table 4. Kinetics of substrate utilisation by *M. c. capripneumoniae* strains expressed as saturation constant Km (µM) values.

Substrate	Km value (µM)								
	19/2	4/2 LC	T3	T7	T9	T11	Baringo	7/1a	G94/83
Glucose	3.5	3.1	5.3	4.6	4.0	6.9	<1	<1	<1
GlcNAc*	1.7	2.5	1.1	1.8	2.5	0.8	<1	<1	<1
Fructose	<1	<1	<1	<1	<1	<1	<1	<1	<1
Glucosamine	116	227	96	142	NT	NT	<1	<1	<1
Mannose	5mM	526	111	122	NT	NT	<1	<1	<1
Pyruvate	13	11	11	12	13	7	14	17	5.8
Lactate	8.1	7.5	14	17	11	18	10	16	4.6
2-oxybutyrate	0.4ǂ	0.4ǂ	2.9mM	830	740	2.2	11	10	9.7
Glycerol	<1	2.1	9.7	7.7	4.5	5.4	3.4	3.5	<1

(<1): not metabolised; (NT): not tested; (ǂ): Substrate metabolised detected at 4 mM but not at 0.4 mM; (*): N-acetylglucosamine.

Table 5. DNA-DNA hybridisation between *M. c. capripneumoniae* strains. Data shown are the mean % hybridisation values from three independent replicate experiments.

M. c. capripneumoniae strains	F38	7/1a	Baringo	4/2 LC
F38	100	NT	NT	78
7/1a	92	100	98	92
Baringo	89	NT	100	93
4/2 LC	84	86	NT	100
19/2	8	83	86	98
Mycoplasma bovis NCTC 10131*	14	9	7	12

(NT): not tested; (*): Included as negative control.

Table 6. Comparison of biochemical types and polymorphism patterns of 16S rRNA genes in *M. capricolum* subsp. *capripneumoniae*.

Strain	Polymorphism pattern*	Biochemical type
F38	IIB	Oxidises organic acids
7/1a	IIB2	Oxidises organic acids
4/2 LC	IIB3	Ferments glucose
19/2	IIB3	Ferments glucose
Baringo	IA	Oxidises organic acids
G1943/80	IA	Oxidises organic acids
G94/83	IA	Oxidises organic acids

(*): Pettersson *et al*. (1998).

Table 7. DNA-DNA hybridisation between *M. c. capripneumoniae* strains (7/1a and 4/2 LC) and other members of the *M. mycoides* cluster. Data shown are the mean % hybridisation values from three independent replicate experiments.

Strain	7/1a	4/2 LC
M. c. capripneumoniae		
7/1a	100	
4/2 LC		100
M. leachii		
PG50 (NCTC 10133)	82	
QR1		98
B144P	77	94
4055	84	91
D318b		88
D424		98
C2306		96
Calf 1		92
M. mycoides subsp. *mycoides*		
PG1 (NCTC 10114)		78
Gladysdale	76	79
4813		79
KH3J	83	77
M. mycoides subsp. *capri*		
Y goat	74	81
LC 1176-2	80	71
G108	70	75
Pendik	75	68
M. bovis (-ve control)		
NCTC 10131	9	12

Furthermore, H_2O_2 has been identified as a pathogenicity factor (via the oxidation of glycerol) in the closely related *M. mycoides* subsp *mycoides* in which the virulent African strains but not the largely non-virulent European strains are strong producers (Houshaymi *et al*., 1997). The Eritrean strains were from a particularly severe outbreak in which mortality rates were particularly high.

Whole genome DNA-DNA hybridisation techniques have been widely used to assess taxonomic relationships amongst cell-walled bacterial groups and have been of fundamental importance in establishing many new species and genera.

They have also been used in a number of studies with mycoplasmas, for example: the classification of spiroplasmas (Townsend *et al*., 1977; Lee and Davis, 1980); the demonstration of the relationship between *M. fermentans* and the organism originally referred to as *M. incognitus* (Saillard *et al*., 1990); the establishment of *M. imitans* as a distinct species, despite its very similar rRNA sequence with *M. gallisepticum* (Johnson, 1984; Dupiellet *et al*., 1988, 1990); the separation of *M. bovis* from the metabolically similar *M. agalactiae* (*M. bovis* was previously regarded as a variety of *M. agalactiae*) (Askaa and Ernø, 1976); and in studies of the *M. mycoides* cluster.

In general, DNA-DNA hybridisation studies have shown a close overall relationship between members of the cluster (Askaa *et al*., 1978; Christiansen and Ernø, 1982; Bonnet *et al*., 1993). However, *M. c. capricolum*, *M. leachii* and *M.* F38-like strains were more closely related to each other than to *M. mycoides* subsp. *capri* strains (International Committee, 1991; Bonnet *et al*., 1993). The close relationship between *M.* F38-like strains and *M. c. capricolum* (70 and 78% under high and low stringency conditions, respectively), led to the renaming of *M.* F38-like strains as *M. c. capripneumoniae* (Bonnet *et al*., 1993; Leach *et al*., 1993).

Mycoplasma Identification is based on the general species definition using a combination of serology and phenotypic data, 16S rRNA gene sequence analyses and DNA–DNA hybridization (Brown *et al*., 2007). The advantage of DNA-DNA hybridisation in taxonomic studies is that it provides an overall indication of the DNA-relatedness of organisms. While 16S rRNA gene sequence analysis is rapid it can provide ambiguous results because mycoplasma species may have up to 100% gene similarity. For instance, the pairs *M. pneumoniae* and *M. genitalium*, *M. mycoides* and *M. c. capricolum* have 98% and 99.8% 16S rRNA sequence similarity, respectively. Serology is also very cumbersome, requiring a large selection of hyperimmune sera and the expertise of few international laboratories. In addition, the serological tests may result in cross-reaction between species because of the high genomic and phenotypic diversity of Mycoplasmas (Thompson *et al*., 2012).

DNA appears particularly suitable in studies of closely related groups, for example the members of the *M. mycoides* cluster (Cottew *et al*., 1987), which show few differences in their 16S rRNA nucleotide sequences (Fox *et al*., 1992). However, compared to many molecular biology techniques, traditional methods for determining DNA-DNA hybridisation are laborious, requiring large quantities of DNA. In addition, detection of hybrid DNA requires that one DNA source is labelled; this was generally achieved by growth of cells using [^{3}H] or [^{14}C]-labelled substrates. Non radio-active labels are of course available today.

In conclusion *M. c. capripneumoniae* strains show a high overall DNA homology, but that nevertheless, the group may be subdivided and the subdivision is consistent with the two biotypes, glucose and organic acid oxidising, determined from biochemical studies. These two groups of strains presumably differ in the possession and/or expression of a substantial range of transport proteins and enzymes associated with sugar metabolism via the Embden-Meyerhoff pathway. Such fundamental metabolic diversity is not usually seen amongst members of the same species and therefore, DNA-DNA hybridisation studies were carried out. The results showed that although the two groups of strains could be separated by their degree of DNA homology, the mean hybridisation value between members of the two groups was 86% well within the value of 70% normally used to indicate separate species. We encourage other molecular biologists to investigate our findings using the many tools available today to see whether further subdivision is possible.

The relatively high DNA homology of *M. c. capripneumoniae* with subsp. *capricolum* has already been reported (Bonnet *et al*., 1993; Christiansen and Ernø, 1982). However, there is also a particularly close relationship between glucose oxidising subsp. *capripneumoniae* strains and *M. leachii* strains.

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Conflict of interest

The authors declare that there is no conflict of interest.

References

Abu-Groun, E.A.M., Taylor, R.R., Varsani, H., Wadher, B.J., Leach, R.J. and Miles, R.J. 1994. Biochemical diversity in the '*M. mycoides'* cluster. Microbiol. 140, 2033-2042.

Askaa, G. and Ernø, C. 1976. Elevation of *Mycoplasma agalactiae* subsp. *bovis* to species rank: *Mycoplasma bovis* (Hale *et al.*) comb. nov. Int. J. Syst. Bacteriol. 26, 323-325.

Askaa, G., Ernø, H. and Ojo, M.O. 1978. Bovine mycoplasmas: classification of groups related to Mycoplasma mycoides. Acta Vet. Scandinavica 19, 166-178.

Bashiruddin, J.B. 1998 Extraction of DNA from mycoplasmas. In: Methods in Molecular Biology: Mycoplasma Protocols. Edited by: R.J. Miles and R.A.J. Nicholas, Humana Press, Totowa, New Jersey, Vol. 104, pp: 141-145.

Bonnet, F., Saillard, C., Bové, J.M., Leach, R.H., Rose, D.L., Cottew, G.S. and Tully, J.G. 1993. DNA relatedness between field isolates of *Mycoplasma* F38 group, the agent of contagious caprine pleuropneumonia, and strains of *Mycoplasma capricolum*. Int. J. Syst. Bacteriol. 43, 597-602.

Brown, D.R., Whitcomb, R.F. and Bradbury, J.M. 2007. Revised minimal standards for description of new species of the class Mollicutes (division Tenericutes). Int. J. Syst. Evol. Microbiol. 57, 2703-2719.

Chingi, A.K., Ernø, H. and Waiyaki, P.G. 1987. Report on serological problems in classification of mycoplasmas from cases of contagious caprine pleuropneumonia in Kenya. In: Agriculture : contagious agalactia and other mycoplasmal diseases of small ruminants. Edited by: G.E. Jones. Office for Official Publications on the European Communities, Luxembourg, pp: 85-89.

Christiansen, C. and Ernø, H. 1982. Classification of the F38 group of caprine mycoplasma strains by DNA hybridization. J. Gen. Microbiol. 128, 2523-2526.

Cottew, G.S., Breard, A., DaMassa, A.J., Ernø, H., Leach, R.H., Lefevre, P.C., Rodwell, A.W. and Smith, G.R. 1987 Review Taxonomy of the *Mycoplasma mycoides* cluster. Isr. J. Med. Sci. 23, 632-635.

Dupiellet, J.-P., Vuillaume, A., Rousselot, D., Bové, J.M. and Bradbury, J.M. 1990. Serological and molecular studies on *Mycoplasma gallisepticum* strains. Zentralblatt Für Bakteriologie Suppl. 20, 859-864.

Dupiellet, J.-P., Vuillaume, A., Rousselot, D., Bové, J.M. and Bradbury, J.M. 1988. Serological and molecular studies on *Mycoplasma gallisepticum* strains. 7th Congress. International Organisation of Mycoplasmology Conference. Abstract, pp: 150.

Fox, G.E, Wisotzkey, J.D. and Jurtshuk Jr, P. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int J. Syst. Bacteriol. 42, 166-170.

Houshaymi, B.M., Miles, R.J. and Nicholas, R.A.J. 1997. Oxidation of glycerol differentiates African from European isolates of *Mycoplasma mycoides* subspecies *mycoides* SC (small colony). Vet. Rec. 140, 182-183.

Houshaymi, B.M., Miles, R.J. and Nicholas, R.A.J. 2002. Studies on strains of *Mycoplasma capricolum* subsp. *capripneumoniae* isolated from outbreaks of contagious caprine pleuropneumonia. Small Rum. Res. 45, 139-143.

International Committee. 1991. International Committee on Systematic Bacteriology-Subcommittee on the Taxonomy of Mollicutes. Int. J. Syst. Bacteriol. 41, 333-336.

Johnson, J.L. 1984. Bacterial classification. III. Nucleic acids in bacterial classification. In *Bergey's Manual of Systematic Bacteriology*. Edited by: N.R. Krieg and J.G. Holt. Williams and Wilkins, Baltimore, Vol. 1, pp: 8-11.

Kanyi Kibe, M., Bidwell, D.E., Turp, P. and Smith, G.R. 1985. Demonstration of cross-reactive antigens in F38 and related mycoplasmas by enzyme linked immunosorbent assay (ELISA) and immunoblotting. J. Hygiene (Cambridge) 95, 95-106.

Leach, R.H., Ernø, H. and MacOwan, K.J. 1993. Proposal for designation of F38-type caprine mycoplasmas as *Mycoplasma capricolum* subsp. *capripneumoniae* subsp. nov. and consequent obligatory relegation of strains currently classified as *M. capricolum* (Tully, Barile, Edward, Theodore, and Ernø 1974) to an additional new subspecies, *M. capricolum* subsp. *capricolum* subsp. nov. Int. J. Syst. Bacteriol. 43, 603-605.

Lee, I.-M. and Davis, R.E. 1980. DNA homology among diverse spiroplasma strains representing several serological groups. Can. J. Microbiol. 26, 1356-1363.

Manso-Silvan, L., Perrier, X. and Thiaucourt, F. 2007. Phylogeny of the *Mycoplasma mycoides* cluster based on analysis of five conserved protein-coding sequences and possible implications for the taxonomy of the group. Int. J. Syst. Evol. Microbiol. 57, 2247-2258.

Miles, R.J., Wadher, B.J., Henderson, C.L. and Mohan, K. 1988. Increased growth yields of *Mycoplasma* species in the presence of pyruvate. Letters App. Microbiol. 7, 149-151.

Miles, R.J. and Agbanyim, C. 1998. Determination of substrate utilisation rates by mycoplasmas. In Methods in Molecular Biology: Mycoplasma Protocols. Edited by R.J. Miles and R.A.J. Nicholas, Humana Press, Totowa, New Jersey, Vol. 104, pp: 95-104.

Pettersson, B., Bölske, G., Thiaucourt, F., Uhlén, M. and Johansson, K.-E. 1998. Molecular evolution of *Mycoplasma capricolum* subsp. *capripneumoniae* strains, based on polymorphisms in the 16S rRNA genes. J. Bacteriol. 180, 2350-2358.

Saillard, C., Carle, P., Bové, J.M., Bébéar, C., Lo, S.-C., Shih, J.W.-K., Wang, R.Y.-H., Rose, D.L. and

Tully, J.G. 1990. Genetic and serologic relatedness between *M. fermentans* strains and mycoplasma recently identified in tissues of AIDS and non-AIDS patients. Res. Virol. 141, 385-395.

Thiaucourt, F., Manso-Silvan, L., Salah, W., Barbe, V., Vacherie, B., Jacob, D., Breton, M., Dupuy, V., Lomenech, A.M., Blanchard, A. and Sirand-Pugnet, P. 2011. Mycoplasma mycoides, from "mycoides Small Colony" to "capri". A microevolutionary perspective. BMC Genomics 12, 114.

Thompson, C.C., Vieira, N.M., Vicente, A.C. and Thompson, F.L. 2012. Towards a genome based taxonomy of Mycoplasmas. Infect. Genet. Evol. 11, 1798-1804.

Townsend, R., Markham, P.G., Plaskitt, A.k. and Daniels, M.J. 1977. Isolation and characterization of Non-Helical strain of *Spiroplasma citri*. J. Gen. Microbiol. 100, 15-21.