Characterization of *Pasteurella* Species Isolated from Lungs of Calves with Pneumonia

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ABSTRACT

During routine bacteriological examination of pneumonic calf lungs it was experienced that many *Pasteurella multocida*-like isolates had a fermentation pattern different from what is generally accepted for *P. multocida sensu stricto*. Forty-one out of 50 strains selected for further investigation were phenotypically related and formed a group of indole-, mannitol- and sorbitol-negative *P. multocida*-like strains, which was tentatively designated taxon 13.

Deoxyribonucleic acid/deoxyribonucleic acid hybridizations including both ornithine positive and ornithine negative strains of taxon 13 allowed the classification of the former as *P. multocida* biovar 6 and the latter as V factor independent strains of *Haemophilus avium*.

Key words: *Pasteurella multocida, Pasteurella* spp., *Haemophilus-Pasteurella-Actinobacillus*, taxonomy, pneumonia.

RÉSUMÉ

L'examen bactériologique routinier des poumons de veaux atteints de pneumonie permit de constater que plusieurs de bactéries semblables à *Pasteurella multocida* affichaient un profil de fermentation différent de celui qui lui est ordinairement propre. Quarante et une des 50 souches choisies pour une étude plus approfondie possédaient un phénotype apparenté et formaient un groupe de souches semblables à *P. multocida* qui réagissaient de façon négative à l'indol, au mannitol et au sorbitol; les auteurs appelèrent provisoirement ces souches: "Taxon 13".

L'hybridation des acides nucléiques des souches du "Taxon 13" qui réagissaient de façon positive à l'ornithine, avec ceux des souches qui y réagissaient de façon négative, permit de classifier les premières comme *P. multocida* biovar #6 et les dernières, comme des souches d'*Haemophilus avium* indépendantes du facteur V.

Mots clés: *Pasteurella multocida, Pasteurella* spp., complexe: *Haemophilus-Pasteurella-Actinobacillus*, taxonomie, pneumonie.

INTRODUCTION

Pasteurella multocida and *Pasteurella haemolytica* are important bacterial pathogens in the respiratory tract of cattle (1).

Significant differences in colonial morphology, biochemical activity, antigenic structure and pathogenicity exist among isolates of *P. multocida*, and different bio- and serotyping systems have been proposed to aid in the epidemiological research (2,3,4,5).

In defiance of these differences it has been advocated for many years to refer to all such strains having a number of basic characters in common as *P. multocida*. This interpretation was confirmed by recent genetic investigations (6) including Frederiksen's biovars 1-6 (2) and Carter's serovars A-E (7). These investigations showed that *P. multocida* was rather homogenous in DNA/DNA hybridizations except for biovars 1 and 6 (6).

During routine examination of strains isolated from pneumonic calf lungs, it was observed that many bovine *P. multocida*-like isolates had a fermentation pattern different from that generally accepted for this species.

The purpose of the present report is to characterize these "atypical" strains and to clarify their taxonomical position.

MATERIALS AND METHODS

The material comprised 61 pneumonic calf lungs submitted to the State Veterinary Serum Laboratory, Copenhagen, for diagnostic examination. They originated from 59 herds distributed over the whole country.

Lung material with pneumonic lesions was inoculated on blood agar (Columbia blood agar base (OXOID) with 5% citrate-stabilized bovine blood), blood agar with polymyxin (12.5 units/mL, NOVO, Denmark), chloral hydrate blood agar (chloral hydrate 1.4 mg/mL), and lactose-sucrose-bromthymolblue agar (modified Drigalski agar) (8). Plates were incubated aerobically and in an atmosphere containing 10% CO_2 at 37°C for 48 hours.

According to preliminary investigations including cultural and biochemical features (formation of indole and production of acid from glucose, lactose, sucrose and salicin) in addition to morphology and staining reactions, eighteen strains of *P. multocida* and 43 strains of *P. multocida*-like organisms were isolated. Seven strains of *P. multocida* and 43 strains of *P. multocida*-

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like organisms totalling 50 strains were selected and kept for further investigations (Table I). Twenty-eight of these were isolated from calves younger than two months of age, the remaining 22 from calves two to nine months old. Twenty-six strains originated from lungs with catarrhal bronchopneumonia, twelve from lungs with fibrinous pneumonia and/or fibrinous pleurisy, and twelve from lungs with chronic purulent lesions and abscess formation.

All 50 strains were phenotypically characterized by standard methods as described previously (9,10). The results were compared to those obtained with reference strains representing recognized species and taxa tentatively assigned to the *Haemophilus-Pasteurella-Actinobacillus* (HPA)-complex (9,11 and Bisgaard, unpublished data). Finally, the isolates were subjected to the hyaluronidase (12) and acriflavine tests (13) both of which are simple indirect methods for identification of type A and D serovars of *P. multocida*.

Two strains representing ornithine positive (K267) and ornithine negative (K117) strains of taxon 13 were selected for genotypical investigations. Determinations of DNA base composition, genome sizes and genetic relatedness were performed as described previously (10).

TABLE I. Identification of Strains Investigated

Organism	Strains	No. of Strains	Type Culture Collection No.
Reference strains			
P. multocida		1	NCTC 10322^T
P. multocida biovar 6	J.E. Smith 182	1	NCTC 11621
Pasteurella sp.	J.E. Smith 166	1	NCTC 11623
Pasteurella sp.	W. Frederiksen P809	1	
H. avium	K.-H. Hinz IPDH2654	1	ATCC 29546^T
Own isolates			
Taxon 13 (orn$^+$, xyl$^+$)	K77, K95, K103, K219, K222, K225, K279, K316, K323, K341, K379, K434, K464, K618, K639, K1931, K1969, K2041, K2091, K2092, K2104	21	
Taxon 13 (orn$^+$, xyl$^-$)	K138, K221, K267, K378 K391, K826, K1238, K1406, K2114, K2139	10	
Taxon 13 (orn$^-$, xyl$^+$)	K117, K200, K223, K332, K343, K417, K461, K1057, K1167, K1997	10	
P. multocida	K424, K664, K875, K1012, K1243, K1404, K1923	7	
Atypical strains belonging to the "HPA-complex"	K432, K673	2	

Abbreviations:
ATCC, American Type Culture Collection, Rockville Maryland, USA;
NCTC, National Collection of Type Cultures, Colindale, London, U.K.;
T, type strain

RESULTS

Phenotypical characters of the 50 strains examined compared to *P. multocida sensu stricto* appear from Table II. Seven strains were diagnosed as *P. multocida*, although differences were noted with respect to fermentation of xylose, lactose and trehalose. Forty-one indole-negative, mannitol- and sorbitol-negative *P. multocida*-like strains formed a group which was tentatively designated taxon 13. This group included 21 strains which could decarboxylate ornithine and produce acid from xylose. Ten strains were ornithine positive and xylose negative, and ten were ornithine negative and xylose positive (Table I). Two strains were found to belong to the HPA-complex, but could not be allocated to any known species or taxa. These strains are not considered further, but their phenotypical characters have been included in Table II to draw attention to the existence of such strains.

Surface cultures of taxon 13 on bovine blood agar were circular, raised and regular with an entire margin. The surface of the colonies was smooth, moist and shiny. The colonies were mucoid and semitransparent with a grayish tinge. A colonical diameter of 2-3 mm was observed after 24 hours aerobic incubation at 37°C. After further incubation for 24 hours flowing margins were observed and the diameter of the colonies had increased by 1-2 mm.

Some characters separating both types of taxon 13 from phenotypically related species and taxa belonging to or tentatively allocated to the HPA-complex are given in Table III.

The results of indirect capsular typing by the acriflavine and hyaluronidase tests showed that all 50 strains belonged to *P. multocida* serovar A, with the exception of four strains which were untypable.

The guanine plus cytosine content (% G + C) of DNA and the molecular weight of the genomes of strains K117 and K267 appear from Table IVA.

Deoxyribonucleic acid relatedness of strains K117 and K267 and some selected species/strains included in or tentatively classed with the family *Pasteurellaceae* Pohl 1981 is given in Table IVB. It appears from Table IVB that strain K267, representing the ornithine positive type of taxon 13 showed 80% DNA homology with NCTC 11621 the reference strain of *P. multocida* biovar 6, and 62% DNA homology with strain P809 a possible other new species within the genus *Pasteurella sensu stricto* (Mutters, unpublished). Strain K267 was related on the 70% DNA binding level with K117 which represents the ornithine negative type of taxon 13. The latter exhibited 81% DNA homology with NCTC 11623, the reference strain for the new *Pasteurella* species consisting of ornithine negative strains similar to *P. multocida* biovar 6 (Mutters, unpublished data). Eighty-eight percent genome relatedness have been obtained between K117 and the type strain of *Haemophilus avium* ATCC 29546^T which is related to the new *Pasteurella* species represented by strain NCTC 11623 on the 80% DNA binding level (Mutters, unpublished results).

TABLE II. Phenotypic Characteristics of Bovine Isolates Tentatively Designated Taxon 13 and Strains K432 and K673 Compared to *P. multocida sensu stricto*

	P. multocida NCTC 10322^T	*P. multocida* (bovine)	Taxon 13	Strain K432	Strain K673
Gram-staining	–	–	–	–	–
Motility, 22°C	–	–	–	–	–
Catalase	+	+	+	+	+
Oxidase, TMPD	+	+	d	w	+
Hugh & Leifson, glucose	F	F	F	F	F
β-hemolysis	–	–0/7[a]	–0/41	–	–
Citrate, Simm.	–	–0/7	–0/41	–	–
Malonate, base	–	–0/7	–0/41	–	–
H_2S/TSI	–	–0/7	–0/41	–	–
KCN, growth	+	+7/7	d6/41	–	–
MR, 37°C	–	–0/7	–0/41	–	–
VP, 37°C	–	–0/7	–0/41	–	–
Nitrate	+	+7/7	+41/41	+	+
Urease	–	–0/7	–0/41	–	–
Arginine dihydr.	–	–0/7	–0/41	–	–
Lysine decarbox.	–	–0/7	–0/41	–	–
Ornithine decarbox.	+	+7/7	d31/41	–	–
Phenylalanine deam.	–	–0/7	–0/41	–	–
Indole	+	+7/7	–0/41	–	–
Phosphatase	+	+7/7	+41/41	+	+
Gelatinase	–	–0/7	–0/41	–	–
Tween 20	–	–0/7	–0/41	–	–
Tween 80	–	–0/7	–0/41	–	–
McConkey, growth	–	–0/7	–0/41	–	+
Pigment	–	–0/7	–0/41	–	–
Glycerol	–	–0/7	–0/41	–	–
Adonitol	–	–0/7	–0/41	–	–
L(+)arabinose	–	–0/7	–0/41	–	–
D(+)xylose	+	d6/7	d31/41	–	+
Dulcitol	–	–0/7	–0/41	–	–
Meso-inositol	–	–0/7	–0/41	–	+
D(–)mannitol	(+)	+7/7	–0/41	+	+
D(–)sorbitol	(+)	+7/7	–0/41	+	+
D(–)fructose	+	+7/7	(+)/+41/41	+	+
D(+)galactose	(+)	+7/7	(+)/+41/41	–	+
D(+)glucose, acid	+	+7/7	(+)/+41/41	+	+
gas	–	–0/7	–0/41	–	–
D(+)mannose	+	+7/7	+/(+)41/41	+	–
L(+)rhamnose	–	–0/7	–0/41	–	–
L(–)sorbose	–	–0/7	–0/41	–	–
Cellobiose	–	–0/7	–0/41	–	–
Lactose	–	d2/7	–0/41	–	(+)
ONPG	–	d2/7	–0/41	–	+
Maltose	–	–0/7	–0/41	+	+
D(+)melibiose	–	–0/7	–0/41	–	–
Sucrose	+	+7/7	(+)/+40/41	+	+
Trehalose	+	d3/7	(+)/+41/41	+	–
D(+)melezitose	–	–0/7	–0/41	–	–
Raffinose	–	–0/7	–0/41	–	(+)
Dextrin	–	–0/7	–0/41	+	+
Inulin	–	–0/7	–0/41	–	–
Amygdalin	–	–0/7	–0/41	–	–
Salicin	–	–0/7	–0/41	–	–
Aesculin hydr.	–	–0/7	–0/41	–	–

\+ : most (90% or more) strains positive within one or two days incubation
– : most (90% or more) strains negative
d : some (less than 90%) strains positive
a : No. of strains positive/No. of strains investigated
w : weak
(+) : positive reaction after three or more days
(+)/+ : majority of reactions delayed (≧ 3 days), some occur within one or two days
+/(+) : majority of reactions occur within one or two days, some reactions delayed

DISCUSSION

Though different species of organisms have been associated with pneumonia in cattle the principal species involved seem to be *P. haemolytica* and *P. multocida* (1). During the present investigation, eighteen strains of *P. multocida* and 43 strains of *P. multocida*-like organisms were isolated (in pure or mixed culture) from 61 cases of pneumonia.

A group of 41 phenotypically related *P. multocida*-like organisms (67%) was tentatively designated taxon 13. Separation of taxon 13 from *P. multocida sensu stricto* was based on differences in indole formation and acid production from mannitol and sorbitol. Strains with phenotypical characters similar to taxon 13 have not been described previously, although considerable phenotypic variability has been reported within *P. multocida* (2,4).

Recent DNA/DNA hybridizations have shown that the mannitol- and sorbitol-negative biovar 6 of *P. multocida* described by Frederiksen (2) does not belong to *P. multocida sensu stricto* (6,14). These investigations have also demonstrated that a phenotypically homogenous group of strains, which deviates from *P. multocida* biovar 6 by the absence of ornithine decarboxylase, is a genetically distinct entity closely related to *H. avium*. According to Mannheim (6) this entity too should be recognized as a new *Pasteurella* species.

A significant degree of phenotypical relatedness was demonstrated between taxon 13, *P. multocida* biovar 6, *H. avium, Pasteurella* sp. strain Smith 221, taxon 1 and *P. gallinarum* (Table III). Differences in indole and trehalose separate ornithine positive strains of taxon 13 from *P. multocida* biovar 6. However, the existence of trehalose positive *P. multocida* biovar 6 has been described by Frederiksen (2). Consequently, ornithine positive strains of taxon 13 can tentatively be regarded as indole negative variants of *P. multocida* biovar 6. Deoxyribonucleic acid binding data confirmed this supposition (Table IVB).

Deviations as to V factor requirement and xylose splitting were observed between ornithine negative strains of taxon 13 and *H. avium*

TABLE III. Some Characteristics Separating Taxon 13 from Phenotypically Related Species and Taxa Belonging to or Tentatively Allocated to the *Haemophilus-Pasteurella-Actinobacillus* Complex

	P. multocida NCTC 10322[T]	*P. multocida* biovar 6 (11)[a]	Taxon 13 Orn[+]	Taxon 13 Orn[-]	*H. avium* ATCC 29546[T] (15)	*Pasteurella* sp. strain Smith 221 (11)	Taxon 1 (9)	*P. gallinarum* (11)	Taxon 4 (9)	*P. pneumotropica*, Henriksen type (11)
V-factor requirement	–	–	–	–	+	–	–	–	–	–
Urease	–	–	–	–	–	–	–	–	–	+
Ornithine	+	+	+/(+)	–	–	–	–	–	–	–
Indole	+	+	–	–	–	+	–	–	–	+
Glycerol	–	–	–	–	ND	–	(+)	–	–	(+)
D(+)xylose	+	–	d	+/(+)	–	–	+	–	–	–
D(–)mannitol	(+)	–	–	–	–	–	+	–	+	–
D(–)sorbitol	(+)	–	–	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	–	–	–	(+)	–
Maltose	–	–	–	–	–	–	–	+	–	+
Trehalose	+	–	+/(+)	+/(+)	+	+	+	+	–	+
Dextrin	–	–	–	–	–	–	–	+	–	+

[a]Figures in brackets are references
ND: Not done

TABLE IVA. G + C Content (mol%) and Genome Sizes (x 10^9d) of Ornithine Positive (K267) and Ornithine Negative (K117) Strains of Taxon 13

	G + C content (mol%)	Genome size (x 10^9d)
K267	42.8	1.9
K117	41.9	1.7

TABLE IVB. DNA Relatedness of Ornithine Positive (K267) and Ornithine Negative (K117) Strains of Taxon 13 and some Selected Species/Strains Included in or Tentatively Classed with the Family *Pasteurellaceae* Pohl 1981

	K267		K117	
	Degree of binding (%)	Standard deviation (%)	Degree of binding (%)	Standard deviation (%)
P. multocida biovar 6 NCTC 11621	80	1.4		
P. sp. K117	69	2.1		
P. sp. SSI P809	62	4.4		
H. avium ATCC 29546[T]			88	1.7
P. sp. NCTC 11623			81	4.2

ATCC 29546[T] (15). V factor requirement has traditionally been considered a generic feature of *Haemophilus*. However, this interpretation has recently been questioned by Mannheim (6). In consequence of that, and due to xylose fermentation seems of little taxonomic value within *Pasteurella sensu stricto* (16), ornithine negative strains of taxon 13 might represent V factor independent strains of *H. avium* ATCC 29546[T]. Deoxyribonucleic acid binding data support this assumption.

All strains but one diagnosed as taxon 13 were markedly affected by the staphylococcal hyaluronidase. In consequence of that, and results discussed previously, routine identification of *P. multocida* based on colonial morphology and a few biochemical tests combined with indirect capsular typing should be highly questioned.

Recently Pohl *et al* (10) suggested to include the Bertschinger and Seifert organism in the species *H. pleuropneumoniae* and to transfer *H. pleuropneumoniae* to the genus *Actinobacillus* Brumpt 1910 as *A. pleuropneumoniae*. The amended species contains a V factor-dependent biovar and a V factor-independent biovar. Similarly, *H. avium* and *P. gallinarum* are phenotypically related and belong to the same DNA homology group, irrespective of their different V factor requirements. *Haemophilus avium* and the ornithine negative strains of taxon 13 might represent a third example, but with different host specificity.

In lungs submitted for routine postmortem examinations no specific lesions have been found which could be correlated with the presence of organisms referable to taxon 13.

The present investigation has shown the need for thorough study of organisms which on routine bacteriological examination appear to be typical *P. multocida*. Although in the present material taxon 13 was not correlated with specific lung lesions, attention will have to be paid to possible ecology and control in relation to infections with such organisms.

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