Pasteurella caballi, a new species from equine clinical specimens

by

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GENERAL INTRODUCTION

The genus *Pasteurella* is currently classified in the family *Pasteurellaceae* together with the related genera *Actinobacillus* and *Haemophilus* (Mannheim, 1984). Members of the genus *Pasteurella* are gram-negative, oxidase-positive, nonmotile, fermentative, rod-shaped bacteria (Carter, 1984; Mutters *et al.*, 1985). All *Pasteurella* species are parasitic on the mucus membranes of the respiratory and digestive tracts of mammals and birds. Under conditions of stress, these organisms can become invasive and play a significant role in the pathogenesis of a variety of infections, including pneumonia, sinusitis, abortion, mastitis, and septicemia (Biberstein, 1981).

It is not uncommon for veterinary microbiologists to isolate organisms that resemble members of the *Pasteurellaceae*, but cannot be identified as one of the recognized species. These isolates may be biochemically atypical strains of known species, or they may represent species that have not yet been described. Very often, a final determination cannot be made without performing genetic studies.

Since 1983, the General Bacteriology Section, National Veterinary Services Laboratories, has received cultures of an aerogenic, gram-negative bacterium that had many characteristics of the genus *Pasteurella* but could not be

assigned to any established species within that genus. These isolates were of particular interest because all were aerogenic and all were isolated from horses, both uncommon characteristics for *Pasteurella*.

The objective of this study was to confirm by physiological and genetic studies that these equine strains constitute a distinct and homogeneous taxon. Biochemical characteristics, guanine plus cytosine contents, and fatty acid profiles were determined and compared to members of *Pasteurellaceae*. Deoxyribonucleic acid relatedness studies were conducted to determine the relationship of these equine strains to each other and to members of the genus *Pasteurella* and other *Pasteurellaceae*.

Explanation of Thesis Format

The following thesis consists of a general introduction, a review of the literature, two separate papers (Sections I and II), and literature cited. The master's candidate, Linda Schlater, is the senior author of the papers and principal investigator of the study.

LITERATURE REVIEW

The genus *Pasteurella* Trevisan 1887 is classified in the family *Pasteurellaceae* Pohl 1981 along with the closely related genera *Actinobacillus* Brumpt 1910 and *Haemophilus* Winslow *et al.* 1917. These organisms are gram-negative, nonmotile, facultatively anaerobic coccobacilli that produce acid from the fermentation of glucose and certain other carbohydrates. They are parasites on the mucous membranes of mammals, birds, and reptiles and, with the exception of *Pasteurella multocida*, are limited to one or two preferred hosts. These organisms are some of the most common and economically significant of animal pathogens (Biberstein, 1981; Mannheim, 1984)

History and Taxonomy

Diseases caused by bacteria now known as *Pasteurella* were recognized and described many years before the causative organisms were isolated and named. Fowl cholera was studied by Chabert in France in 1780, one hundred years before the etiologic agent was cultured (Gray, 1913). An accurate description of the clinical signs of fowl cholera was given in 1867 in Washington, Iowa, where losses had occurred in chickens, turkeys, and geese (Anon., 1867).

The first descriptions of organisms of the genus *Pasteurella* were made in 1877 and 1878 when both Perroncito

in Italy and Semmer in Russia described a microbe "having a rounded form, and appearing singly, or two combined" in material from chickens with fowl cholera (Gray, 1913).

In 1880, Toussaint and Pasteur isolated and characterized the etiologic agent. Pasteur also discovered that the fowl cholera agent could be attenuated by artificial culture and act as a vaccine against virulent natural infection (Gray, 1913).

In 1883 Burrill gave the first of many names to the fowl cholera agent, *Micrococcus gallicidus* (Fredericksen, 1989). Two years later, Kitt studied the organisms that caused fowl cholera, swine plague, and the septicemias of cattle, swine, sheep, goats, horses, and deer. He concluded the organisms were the same and named them *Bacterium bipolare-multocidum*. The name *Bacterium septicaemia-hemorrhagica* was used in 1886 by Hueppe for the etiologic agents of fowl cholera, rabbit, cattle and swine septicemia (Rosenbusch and Merchant, 1939).

In 1887 Trevisan created the genus *Pasteurella* for the fowl cholera agent, which he named *Pasteurella cholerae-gallinarum*, and similar microorganisms. Two years later, Trevisan described 12 species of pasteurellae that he had classified on the basis of lesions produced in different animals, the bipolar staining of the microorganisms, or the name of the author who had described the disease or

etiologic agent (Zinneman, 1981). The taxonomy of the genus was revised in 1897 when Flugge used animal origin as a basis for naming isolates, i.e., *P. aviseptica, P. boviseptica, P. suiseptica,* and *P. muriseptica* (Carter, 1967).

The zoological subdivision of the pasteurellae remained popular for some years, in part because Lignieres used it when describing the animal diseases, or "pasteurelloses", caused by these organisms (Gray, 1913). Early in this century, several investigators reported that there were few differences in the pasteurellae isolated from the different species of animals, and, as a result, Topley and Wilson (1929) proposed that these names be combined as *Pasteurella septica.*

At Iowa State University, Rosenbusch and Merchant (1939) studied 114 strains isolated from hemorrhagic septicemia in animals. They concluded that the organisms formed two species, a nonhemolytic, indole-forming organism for which they proposed the name *Pasteurella multocida* and a hemolytic, indole-negative organism from cattle and sheep that had been described by Newsom and Cross (1932) and named *Pasteurella haemolytica.* *Pasteurella multocida* was widely accepted and in 1985 it was officially recognized by the Judicial Commission (1985) as the name of the type species of the genus *Pasteurella.*

From 1950 until 1982, five additional species were added to the genus *Pasteurella* based on their phenotypic similarities to *P. multocida*; namely, *P. pneumotropica* (Jawetz, 1950), *P. gallinarum* (Hall *et al.*, 1955), *P. ureae* (Jones, 1962), *P. aerogenes* (McAllister and Carter, 1974), and *P. testudinis* (Snipes and Biberstein, 1982).

In addition to the validly published species, a variety of additional taxa were tentatively assigned to the genus *Pasteurella*. These included *Pasteurella* "gas" or *Pasteurella* new species 1 (Gump and Holden, 1972), the bovine lymphangitidis group (Jayaraman and Suthumadhaven, 1974), a *Pasteurella haemolytica*-like organism from porcine pleuropneumonia (Mannheim, 1981), *Pasteurella* species "Mair" (Mannheim, 1981), "*Pasteurella bettii*" (Mannheim, 1981), as well as several unnamed taxa from poultry and other animals (Bisgaard, 1982).

Various investigators reported on the difficulty in distinguishing the recognized species of *Pasteurella* and *Pasteurella*-like taxa from the genus *Actinobacillus*. For example, Mráz (1969) proposed transferring *P. haemolytica* to the genus *Actinobacillus* because of that organism's phenotypic similarity to *A. lignieresii*, the type strain of *Actinobacillus*. A similar proposal was made by Fredericksen (1973) in regard to *P. ureae*. Hatt and coworkers (1978) at the American Type Culture Collection presented a phenotypic

comparison of *Pasteurella* and *Actinobacillus* and concluded that the two genera could not be differentiated from each other by conventional biochemical methods.

In the early 1980s, researchers began to use DNA-DNA hybridization studies to establish genetic relationships among members of the *Pasteurellaceae*. The DNA-DNA hybridization technique used in these studies was the renaturation method of De Ley *et al.* (1970) which detects reassociation of single stranded DNAs in free solution under stringent conditions. Reassociation was measured optically as a decrease in absorbency at 260 nm and expressed as percent relatedness of the DNAs. Strains exhibiting relatedness above 85% were considered the same species and those linked by DNA relatedness above 50% represented a genus (Mutters *et al.*, 1985, 1989). Based on these criteria, a new classification of the *Pasteurellaceae* and the genus *Pasteurella* has emerged (Pohl, 1981; Mutters *et al.*, 1985).

The genus *Pasteurella* sensu stricto as proposed by Mutters *et al.* (1985) includes *P. multocida* (which has been divided into three subspecies), *P. gallinarum*, and nine new species. The species of the genus *Pasteurella* sensu stricto, their usual hosts, and any previous designations are given in Table 1.

Table 1. Taxa classified in the genus *Pasteurella* sensu stricto[a]

Species	Main hosts	Previous designations
P. multocida		
subsp. *multocida*	mammals, man, birds	
subsp. *septica*	mammals, man, birds	
subsp. *gallicida*	birds	
P. dagmatis	man, dog, cat	*P. pneumontropica* biotype Henriksen, *Pasteurella* "gas" *Pasteurella* new species 1
P. gallinarum	chicken	
P. volantium	chicken, man	*H. avium*, arabinose (-), ONPG (+), mannitol (+), maltose (+)
P. species A	fowl	*H. avium*, arabinose (+)
P. canis		
biotype 1	dog, man	*P. multocida* "dog type" strains
biotype 2	calf	Bisgaard taxon 13, ornithine (+)
P. stomatis	dog, cat, man	

[a]Data taken from Mutters *et al.*, 1989.

Table 1. (continued)

Species	Main hosts	Previous designations
P. avium NAD-requiring	chicken	*H. avium*, arabinose (-), ONPG (-), mannitol (-), maltose (-)
NAD-independent	calf	Bisgaard taxon 13, ornithine (-)
P. species B	dog, cat, man	*P. multocida*, biotype 6, dulcitol (+)
P. langaa	chicken	Bisgaard taxon 4
P. anatis	duck	Bisgaard taxon 1

Taxa excluded from *Pasteurella* on the basis of genetic studies include *P. haemolytica* biovars A and T, *P. ureae*, the *P. haemolytica*-like organism of Bertschinger and Seifert, *P. testudinis*, *P. aerogenes*, *P.* species "Mair", and the Jawetz and Heyl biotypes of *P. pneumotropica* (Pohl, 1981; Mutters *et al.*, 1985). *P. ureae* and the Bertschinger and Seifert organism were found to be closely related to the type strain of *Actinobacillus* and have been transferred to that genus as *A. ureae* and *A. pleuro- pneumoniae*, respectively (Pohl, *et al.* 1983; Mutters *et al.*, 1986a). Genetic analyses indicate that *P. haemolytica* biovar A and

P. testudinis may form a new genus in the family *Pasteurellaceae*. New names have not yet been proposed for these taxa pending further studies (Mutters *et al.*, 1986b). The exact taxonomic positions of *P. pneumo- tropica* biotypes Jawetz and Heyl and *P. aerogenes* are still being investigated, but they do not belong in the genus *Pasteurella* (Mutters *et al.*, 1985). The status of "*P. bettii*", *P. haemolytica* biovar T, *P.* species "Mair", and the bovine lymphangitidis group is unresolved. As a result of their DNA studies, Mutters *et al.* (1985) concluded that these organisms did not belong in *Pasteurella*. Sneath and Stevens (1990), however, recently proposed that these taxa be placed in the genus *Pasteurella* as *P. bettii*, *P. trehalosi*, *P. mairi*, and *P. lymphangitidis* based on results of a numerical taxonomic study. Because there is no "official" classification, acceptance and usage by the scientific community will determine the ultimate classification of these organisms.

Hosts

The pasteurellae are parasitic on the mucous membranes of the upper respiratory and digestive tracts of various species of animals. *Pasteurella multocida* is widespread throughout the world and has been isolated from more than

one hundred species of wild and domestic mammals and birds (Biberstein, 1979; Rosen, 1981; Wallach and Boever, 1983).

In contrast to *P. multocida*, the other species in the genus *Pasteurella* are limited to one or two preferred hosts. *Pasteurella dagmatis, P. canis, P. stomatis*, and the unnamed taxon *P.* species B are commensals of the mouth and respiratory tract of dogs and cats. Domestic fowl are the natural hosts of *P. gallinarum, P. avium, P. volantium, P. anatis, P. langaa*, and *P.* species A (Mutters et al., 1985). *Pasteurella trehalosi* (formerly *P. haemolytica* biotype T) is associated with sheep. The natural habitats of *P. bettii, P. lymphangitidis,* and *P. mairi* have not been fully determined (Sneath and Stevens, 1990).

Equine Pasteurellosis

Reports of pasteurellosis in horses are relatively uncommon. Hemorrhagic septicemia caused by *P. multocida*, type B, is occasionally reported among horses and donkeys in India (Pavri and Apte, 1967). *P. haemolytica* has been isolated from sporadic cases of neonatal septicemia (Peet *et al.*, 1977), abortion (Webb *et al.*, 1980), and ulcerative lymphangitis (Miller and Dresher, 1981). *P. haemolytica* was also incriminated in the deaths of more than 7000 horses and donkeys in Mexico (Valdes, 1963).

Reports of *P. haemolytica* from horses or other nonruminant sources are subject to question. Biberstein (1978) has reported that organisms described in the literature as *P. haemolytica* that were isolated from nonruminant hosts differ from ruminant strains in a number of biochemical and serological characters and probably do not belong to the species *P. haemolytica*. Unfortunately, none of the *P. haemolytica* strains associated with the reports cited above were fully characterized or confirmed by a reference laboratory and their identity remains uncertain.

Morphology and Growth

Members of the genus *Pasteurella* are gram-negative, nonmotile, coccoid to rod-shaped organisms, 0.3-1.0 μm in diameter and 1.0-2.0 μm in length. Cells are arranged singly or, less frequently, in pairs or short chains. Filamentous forms may be seen as strains are subcultured (Carter, 1984). Bipolar staining is often observed, especially in preparations made from fresh tissue stained with a Romanowsky-type stain, such as Wright or Giemsa (Biberstein, 1981). Endospores are not produced and cells are not acid fast. Capsules are produced by most of the virulent strains of *P. multocida*. Growth occurs at temperatures ranging from 22-44°C, with an optimum of 37°C. *Pasteurella* species are facultative anaerobes; they grow

well within 24h either aerobically or in 5-10% carbon dioxide (Carter, 1984).

Pasteurellae grow readily on blood agar, forming smooth, circular, grayish or yellowish colonies, 1-3 mm in diameter, after overnight incubation at 37°C. Mucoid strains of *P. multocida* produce moist, often confluent colonies (Carter, 1984; Mutters *et al.*, 1985). *Pasteurella mairi* and *P. trehalosi* produce distinct zones of beta hemolysis on sheep blood agar (Sneath and Stevens, 1990).

Pasteurella species are alkaline phosphatase and oxidase positive. Catalase is almost always positive. Nitrate is reduced to nitrite. The genus is methyl red- and Voges-Proskauer-negative. Tests for arginine dihydrolase, lysine decarboxylase, and liquefaction of gelatin are negative. Acid is produced from glucose and certain other fermentable compounds. With the exception of *P. dagmatis* and *P. bettii*, the pasteurellae do not produce gas from the fermentation of carbohydrates (Mutters *et al.*, 1985; Sneath and Stevens, 1990).

Taxonomy, Classification, and Nomenclature

Taxonomy has been defined as the science of classification, including its basis, principles, procedures, and rules (Simpson, 1961). Classification, nomenclature, and identification are the three interrelated areas of

taxonomy. Classification is the orderly arrangement of organisms into groups (taxa) on the basis of their relationships to one another. Nomenclature is the assignment of names to the taxa according to international rules. Identification is the process of assigning an isolate to the correct group (Staley and Krieg, 1984).

The primary purpose of classification is to distinguish one organism from another and to group similar organisms on the basis of criteria that facilitate their recognition. The purpose of nomenclature is to provide a convenient system of communication to define an organism without having to list its characteristics (Brenner, 1983). Species are named according to rules and recommendations as set forth in the International Code of Bacterial Nomenclature (Lapage *et al.*, 1976). These rules ensure that bacterial names are formed the same way so that each name has the same meaning to everyone.

Identification is the practical use of classification and nomenclature to identify causative organisms of disease, to distinguish desirable organisms from undesirable ones, to verify the authenticity of an isolate, or to recognize new taxa (Brenner, 1985; Staley and Krieg, 1984).

The bacterial species

The basic taxonomic group in the classification of bacteria is the species. A species is defined on the basis of (1) structural traits of shape, size, mode of movement, staining reactions, and growth characteristics, (2) biochemical and nutritional traits, end products, and cell components, (3) physiologic traits relative to oxygen, temperature, pH, and response to antimicrobials, (4) ecologic traits, and (5) deoxyribonucleotide (DNA) base composition and relatedness (Joklik *et al.*, 1988). One strain of a species (often the first strain isolated) is chosen arbitrarily to represent the species. It is designated the type strain and serves as the reference specimen for the species (Staley and Krieg, 1984).

Genetic Studies

For many years, bacterial classification schemes were built upon a variety of phenotypic characteristics. Strains were assigned to groups based on morphology, staining properties, growth requirements, biochemistry, host range, and pathogenicity. Although successful in many respects, this method was not precise enough for distinguishing superficially similar organisms or for determining phylogenetic relatedness (Johnson, 1984).

In the early 1960s, researchers began to develop methods for the direct analysis of the chromosomal DNA of microbial cells. This approach provided more information on the genetic relationships of bacteria because the constituent molecules of the genes could be studied rather than the products of their expression. The two methods most widely used for this purpose are the determination of overall base composition of the DNA and the comparison of base sequence similarities obtained by in vitro DNA-DNA hybridization studies (Owen and Pitcher, 1985).

DNA base composition

DNA base composition is expressed as the mole percent guanine plus cytosine (mol% G+C) content. The mol% G+C content in bacterial DNA ranges from about 25 to 75% and closely related bacteria have similar G+C values. Members of the same species should not differ more than 2% in mean G+C content and members of the same genus should have a G+C content which differs by no more than 5% (Owen and Pitcher, 1985). It is important to recognize that two organisms with similar mol% G+C content are not necessarily closely related. (DNAs from humans and *Bacillus subtilis* have similar G+C contents.) This is because the mol% G+C values do not take into account the nucleotide sequences of the DNA (Johnson, 1984).

DNA reassociation

DNA has several unique characteristics that allow us to determine similarities in the linear arrangement of the nucleotide bases. DNA is composed of two strands of the deoxyribonucleotide bases adenine (A), thymine (T), guanine (G), and cytosine (C). The two strands are held together by hydrogen bonds that are formed only between A and T and between G and C. The strands are complementary; if there is a guanine on one strand, a cytosine is directly across from it on the other strand. Complementary strands of DNA can be separated by heat or by treatment with alkali. When incubated at the proper temperature, the separated strands will reassociate. Single-stranded DNA will reassociate with a complementary DNA strand from the same or a different organism. This ability to dissociate DNA strands and allow them to reassociate specifically with nucleic acids from a homologous or heterologous source is the basis for all nucleic acid studies (Brenner, 1978).

Base sequence similarities between two organisms are determined by DNA-DNA hybridization and by thermal stability studies. Hybridization studies measure the fraction of the genome of one organism that can reassociate with DNA from another organism under specfic conditions of ionic strength and temperature. Results are expressed as percent relatedness. Thermal stability studies are used to estimate

the degree of base mispairing in heterologous duplexes (hybrids). DNA hybrids with a high degree of base-pair matching require more heat for denaturation. Results of thermal stability studies are expressed as percent divergence (Johnson, 1981).

There are two basic techniques for DNA-DNA hybridization studies: membrane filter and free-solution hybridization. The membrane filter method involves immobilizing denatured, unlabeled DNA to nitrocellulose membrane filters. The bound DNA is hybridized with a radiolabeled DNA probe. After washing, the amount of duplex formation is estimated by scintillation counting of the filter.

Reassociation of DNA in solution may be monitored optically by ultraviolet spectrophotometry, or it may be measured by incubating a small amount of sheared, denatured, radioactively labeled DNA with an excess of unlabeled, denatured DNA. In the latter method, duplex formation is assayed by adsorption to hydroxylapatite or by resistance to S1 nuclease (Johnson, 1981).

DNA reassociation experiments provide an overall measurement of genetic similarity between organisms, which reflects their past history. Thus DNA relatedness values represent phylogenetic relationships. Since hybridization experiments are independent of growth media or growth

conditions, they provide relatedness values that have the same meaning for all groups of organisms (Johnson, 1987).

There is general agreement that a genetic species consists of strains that are 70% or more related at optimal reassociation conditions and 55% or more related at less than optimal conditions and whose DNAs contain 6% or less divergence in related sequences (Brenner, 1981). The 70% species relatedness rule has occasionally been ignored when existing nomenclature is accepted and changes would cause considerable confusion. For example, *E. coli* and the four species of *Shigella* are all 70% or more related and should be grouped in the same species. This change has not been proposed in order to avoid confusion among members of the medical community (Brenner, 1985).

At this time, there is no genetic definition of a genus that is applicable to all groups of bacteria. In general, a genus should consist of phenotypically similar strains that are more closely related to each other than to species of other genera (Farmer *et al.*, 1980).

Although DNA relatedness experiments have become an integral part of bacterial taxonomic studies, the importance of phenotypic characterization should not be overlooked. It is usually on the basis of unusual or atypical phenotypic properties that strains are selected for DNA studies. After the genetic characterization of a species has been

completed, it is important to reexamine the biochemical properties and select those tests that reflect the genetic relationships and are of diagnostic value for identification of the species in the laboratory (MacInnes and Borr, 1990).

SECTION I. AN AEROGENIC *PASTEURELLA*-LIKE ORGANISM ISOLATED FROM HORSES

An aerogenic *Pasteurella*-like organism isolated from horses

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SUMMARY

Thirteen strains of a gram-negative, fermentative bacterium that produced gas from glucose were isolated from horses with a variety of clinical conditions. The morphological and biochemical characteristics of this bacterium are similar to those described for the family *Pasteurellaceae*. These strains appear to constitute a new taxon within the genus *Pasteurella*; however, the final taxonomic position of this group depends upon more detailed genetic studies. Case histories indicate that this bacterium may be a primary respiratory pathogen and may play a secondary role in various other disease conditions.

INTRODUCTION

Members of the genus *Pasteurella* are commonly isolated from a variety of animal species including cattle, sheep, swine, poultry, dogs, cats, and rabbits, but are rarely isolated from horses. Hemorrhagic septicemia caused by *Pasteurella multocida* has been reported among horses and donkeys in India (Pavri and Apte, 1967). *Pasteurella haemolytica* has been isolated from horses in cases of ulcerative lymphangitis, abortion, and neonatal septicemia (Peet *et al.*, 1977; Webb *et al.*, 1980; Miller and Dresher, 1981). *Pasteurella haemolytica* has also been incriminated in an epizootic disease in Mexico that resulted in the death of more than 7,000 horses and donkeys (Valdes, 1963). This report describes the characteristics of an aerogenic *Pasteurella*-like organism isolated from horses.

MATERIALS AND METHODS

Bacterial Strains

Eleven of the 13 strains examined were isolated at various state diagnostic laboratories and submitted to the National Veterinary Services Laboratories (NVSL) for identification. Two strains were isolated at the NVSL from swabs (1 uterine, 1 leg wound) submitted in transport media. The state of origin, specific source of isolation, and any available clinical information are given in Table 1 for each strain studied.

Identification Methods

Cell size was measured with a light microscope after staining with Kopeloff's Gram stain method (Cottral, 1978). Motility was determined by examination of wet-mount preparations using phase-contrast microscopy. Wet mounts examined for motility were prepared using growth from chocolate agar (Columbia base; Difco Laboratories, Detroit, Michigan) slants that had been incubated for 24h at 25°C. Colony morphology was determined by observing 24h growth on bovine blood agar. Growth from a blood agar plate was scraped up with an inoculating loop and examined for the presence of pigment. Anaerobic growth was determined by incubating blood agar plates in an anaerobic glove box

Table 1. Sources of aerogenic *Pasteurella*-like strains of equine origin

Strain ID	State	Source	Additional clinical and/or pathological findings
84-851	WA	Uterus	None
84-314	CA	Lung abscess	Acute respiratory signs
84-513	NM	Tracheal wash	None
84-532	SC	Uterus	Metritis; *Streptococcus zooepidemicus* and *Staphylococcus aureus* also isolated
84-679	IN	Pleural fluid	Severe pleuritis and chronic active necrotizing broncho-pneumonia; organism isolated in high numbers
84-687	WA	Uterus	Pyometra
85-256	KY	Gutteral pouch	Severe sinus infection
85-683	MD	Cervix	Mare unable to conceive; *Streptococcus zooepidemicus* also isolated
86-026	PA	Third metacarpal	Osteomyelitis; *Actinomyces* sp. also isolated
86-200	MT	Leg wound	Numerous contaminants also isolated

Table 1. (continued)

Strain ID	State	Source	Additional clinical and/or pathological findings
86-514	WI	Lung	Pneumonia; fungus also isolated
87-127	IL	Lung	Inhalation pneumonia secondary to esophageal ectasia
87-151	CA	Lung	Chronic respiratory problem; euthanized after sudden onset of ataxia

(Forma Scientific, Marietta, Ohio). Carbohydrate utilization was determined using heart infusion broth (Difco) with 1% carbohydrate and Andrade's indicator. Cultures were observed daily for 1 week for acid production. An inverted Durham tube was placed in the glucose tube to detect gas production. Presence of catalase was tested by adding 3% H_2O_2 to 24h colonies on chocolate agar slants and observing for production of bubbles. The presence of indophenol oxidase was determined using a commercially available reagent (Cepti-Seal; Marion Scientific Corporation, Kansas City, Missouri). Tests for the glycosidase enzymes were performed as described Kilian and Bulow (1976). Tests for aesculin hydrolysis, amino acid decarboxylases (Moeller formula), indole (Ehrlich's method), gelatin liquefaction, nitrate, nitrite, triple sugar iron,

and urease (Christensen method) were performed by standard techniques using commercially available dehydrated media whenever possible (MacFaddin, 1980). The nitrate and nitrite broths were supplemented with 5% bovine serum. All biochemical tests were incubated aerobically at 37°C.

Antimicrobial Susceptibility Tests

Antimicrobial agent susceptibility was determined by a standard technique (Bauer *et al.*, 1966) using Mueller-Hinton agar (Difco) supplemented with 5% sheep blood and 1% IsoVitaleX (Baltimore Biological Laboratories, Cockeysville, Maryland). The following antimicrobial disks (Difco) were used: ampicillin, 10 μg; cephalothin, 30 μg; chloramphenicol, 30 μg; erythromycin, 15 μg; gentamicin, 10 μg; kanamycin, 30 μg; nalidixic acid, 30 μg; neomycin, 30 μg; penicillin G, 10 U; streptomycin, 10 μg; sulfadiazine, 300 μg; tetracycline, 30 μg; tobramycin, 10 μg; and trimethoprim, 5 μg.

RESULTS

Cellular and Colony Characteristics

Examination of stained smears of all strains revealed gram-negative bacilli, 1.0-1.6 μm in length, arranged singly or in pairs. Most cells showed bipolar staining. All strains were nonmotile. After 24h incubation, colonies on blood agar were 1.0-1.5 mm in diameter, round, slightly raised, smooth, shiny grayish-yellow in color, and non-adherent. No hemolysis was observed. Pigment ranged from cream (4/13 strains) to lemon yellow (9/13 strains) in color. All cultures grew well aerobically and anaerobically.

Biochemical Characteristics

The phenotypic characteristics of the equine strains studied are given in Table 2. All strains were positive for cytochrome oxidase, ß-galactosidase, and nitrate reduction. Seven of 13 strains were positive for ornithine decarboxylase. All isolates were negative for acetoin, aesculin hydrolysis, arginine decarboxylase, catalase, α-fucosidase, gelatin liquefaction, ß-glucuronidase, indole, lysine decarboxylase, nitrite reduction, and urease. All strains produced acid and gas from D-glucose and acid from fructose, D-galactose, maltose, D-mannose, and sucrose within 48 hr. Fermentation of lactose was delayed for all

Table 2. Phenotypic characteristics of 13 aerogenic *Pasteurella*-like bacteria isolated from horses.

Test	Usual result[a]	% positive
Acid from adonitol	-	0
Acid from L-arabinose	-	0
Acid from cellobiose	-	0
Acid from dulcitol	-	0
Acid from fructose	+	100
Acid from D-galactose	+	100
Acid from D-glucose	+	100
Acid from *myo*-inositol	-	8
Acid from lactose	(+)	100
Acid from maltose	+	100
Acid from D-mannitol	+	92
Acid from mannose	+	100
Acid from raffinose	d	69
Acid from L-rhamnose	-	8
Acid from salicin	-	0
Acid from D-sorbitol	-	0
Acid from sucrose	+	100
Acid from trehalose	-	0
Acid from D-xylose	d	61
TSI slant/butt	Acid/acid	100
TSI gas/H_2S	-/-	0
Motility (25°C)	-	0
Catalase	-	0
Oxidase	+	100
Nitrate reduction	+	100
Nitrite reduction	-	0
Indole	-	0
Urease	-	0
Gelatinase	-	0
Aesculin hydrolysis	-	0

[a] + = 90% or more positive in 1 or 2 days; - = no reaction, 90% or more; (+) = 90% or more positive after 3 or more days; d = more than 10% and less than 90% positive at 2-7 days).

Table 2. (continued)

Test	Usual result	% positive
Lysine decarboxylase	-	0
Arginine decarboxylase	-	0
Ornithine decarboxylase	d	61
ß-galactosidase	+	100
ß-glucuronidase	-	0
α-fucosidase	-	0
MacConkey agar	Scant or no growth	0
ß-hemolysis	-	0
Yellow pigment	d	69

strains. Variable results were observed for *myo*-inositol (1/13 strains), D-mannitol (12/13 strains), raffinose 9/13 strains), L-rhamnose (1/13 strains), and D-xylose (8/13 strains). Those strains producing acid from raffinose did so after 2 days. None of the strains produced acid from adonitol, L-arabinose, cellobiose, dulcitol, salicin, D-sorbitol, or trehalose.

Antimicrobial Susceptibility

Each of the 13 strains tested was susceptible to ampicillin, cephalothin, chloramphenicol, erythromycin, gentamicin, kanamycin, nalidixic acid, penicillin G, tetracycline, tobramycin, and trimethoprim. Intermediate zones of susceptibility to neomycin (8/13) and streptomycin (4/13) were observed for some strains. The remaining

strains were susceptible to neomycin and streptomycin. All of the strains were resistant to sulfadiazine.

DISCUSSION

The 13 equine strains studied showed characteristics typical of the family *Pasteurellaceae* (Mannheim, 1984). They were gram-negative, nonmotile, coccoid to rod-shaped, facultatively anaerobic, and they produced acid from carbohydrates. Of the three existing genera (*Actinobacillus, Haemophilus, Pasteurella*), the phenotypic characteristics observed for these isolates most closely resemble those described for *Pasteurella* (Carter, 1984). Only the negative catalase test is not typical of that genus. The lack of dependency on X or V factor would exclude the genus *Haemophilus* (Kilian and Biberstein, 1984). Scant or no growth on MacConkey agar, absence of sticky colonies, and a negative urease test are not typical characteristics for actinobacilli (Phillips, 1984).

Gas production from glucose or other carbohydrates is unusual for a *Pasteurella* species. The current edition of *Bergey's Manual of Systematic Bacteriology* lists only 1 species, *Pasteurella aerogenes*, that produces gas from carbohydrates (Carter, 1984). *Pasteurella aerogenes* has been isolated from swine, rabbit, cattle, and human sources (McAllister and Carter, 1974; Bercovier *et al.*, 1981; Weaver *et al.*, 1985). A second aerogenic species, *Pasteurella dagmatis* has been proposed to include those isolates formerly referred to as *Pasteurella* new species 1

"gas" and *Pasteurella pneumotropica* biovar Henriksen (Mutters *et al.*, 1985). *Pasteurella dagmatis* has been isolated from dogs and cats and from human infections resulting from dog and cat bite wounds (Weaver *et al.*, 1985). The biochemical characteristics of the equine isolates reported here differ from those described for both *P. aerogenes* and *P. dagmatis* (Table 3). These equine isolates appear to represent a new taxon in the genus *Pasteurella*. However, before a name can be proposed for this group, genetic studies need to be conducted to confirm its taxonomic position.

Table 3. Differential biochemical characteristics of aerogenic *Pasteurella*-like strains of equine origin and strains of *Pasteurella aerogenes* and *Pasteurella dagmatis*

Test	*P. aerogenes*[a]	*P. dagmatis*	Equine isolates
Acid from L-arabinose	+	-	-
Acid from D-mannitol	-	-	+
Acid from raffinose	-	-	d[b]
Acid from trehalose	-	+	-
Acid from D-xylose	+	-	d
Catalase	+	+	-
Indole	-	+	-
Urease	+	+	-
Ornithine decarboxylase	+	-	d

[a]Data for previously described species adapted from Bercovier *et al.*, 1981; Carter, 1984; and Mutters *et al.*, 1985.

[b]d, Strains vary within species.

The role of this group of organisms in equine disease needs to be clarified. The organism was isolated in large numbers and in relatively pure culture from horses with clinical and/or pathologic evidence of respiratory disease, suggesting a primary pathogenic role in these conditions. The pathogenicity of the organism in cases of metritis and osteomyelitis is uncertain because known pathogens were also isolated (Table 1). These findings are not surprising because other members of the family *Pasteurellaceae* are normal inhabitants of mucous membranes of the respiratory and genital tracts and are capable of causing primary as well as opportunistic secondary infections.

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SECTION II. *PASTEURELLA CABALLI*, A NEW SPECIES FROM EQUINE CLINICAL SPECIMENS

Pasteurella caballi, a new species from equine clinical specimens

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SUMMARY

The name *Pasteurella caballi* sp. nov. is proposed for a group of organisms represented by 29 strains isolated from respiratory and other infections in horses. *Pasteurella caballi* strains are gram-negative, oxidase-positive, nonmotile, fermentative rods with the key characteristics of the genus *Pasteurella*. These strains differed from other *Pasteurella* species in that all were aerogenic and catalase-negative, and some strains produced acid from *myo*-inositol and L-rhamnose. The levels of deoxyribonucleic acid (DNA) relatedness of 28 *P. caballi* strains with labeled DNA from the proposed type strain averaged 91% and 85% (hydroxylapatite method at 55°C and 70°C). *Pasteurella caballi* was 13 to 53% related to strains representing 22 other species of *Pasteurellaceae*. The guanine-plus-cytosine content of the DNA of four *P. caballi* strains was 41 to 42 mol%. The type strain is 83851 (=ATCC 49197).

INTRODUCTION

The genus *Pasteurella* is classified in the family *Pasteurellaceae* together with the related genera *Actinobacillus* and *Haemophilus*. Members of the genus *Pasteurella* are gram-negative, oxidase-positive, nonmotile, fermentative, rod-shaped bacteria (Carter, 1984; Mutters *et al.*, 1985). All *Pasteurella* species are parasitic on the mucus membranes of the respiratory and digestive tracts of mammals and birds. Under conditions of stress, these organisms can become invasive and play a significant role in the pathogenesis of a variety of infections, including pneumonia, sinusitis, abortion, mastitis, and septicemia (Biberstein, 1981).

Since 1983, the General Bacteriology Section, National Veterinary Services Laboratories, has received cultures of an aerogenic, gram-negative bacterium that had many characteristics of the genus *Pasteurella* but could not be assigned to any established species within the genus. Our attention was originally drawn to these strains because they were all isolated from horses, an uncommon source for *Pasteurella*. In a previous report, the biochemical characteristics of 13 strains were described (Schlater, 1989), but since no DNA hybridization information was available, it was not possible to properly classify them. Subsequent biochemical and genetic studies on these 13, as

well as 16 additional strains, showed that the aerogenic equine strains were genetically similar to each other but distinct from other phenotypically similar *Pasteurellaceae*, thereby warranting their designation as a new species, *Pasteurella caballi*.

MATERIALS AND METHODS

Bacterial Strains

The 29 *P. caballi* strains studied and their sources are given in Table 1. Strains representative of species within the family *Pasteurellaceae* used in the DNA hybridization studies were from the sources listed in Table 2. Stock cultures of *P. caballi* and reference strains were stored at -70°C in 0.4ml aliquots of defibrinated rabbit blood. Isolates were subcultured twice on blood agar (37°C, 18h) before biochemical testing.

Morphology of Colonies and Cells

Colonial morphology was determined by observing 24h growth on blood agar (heart infusion agar base; Difco Laboratories, Detroit, Mich.) containing 5% defibrinated bovine blood. Growth from the blood agar plate was removed with an inoculating loop and examined against white filter paper for the presence of pigment. Hemolytic activity was determined on bovine, sheep, and rabbit blood agar. Gram staining and acid-fast staining were done by the methods of Kopeloff and Ziehl-Neelson, respectively, on smears prepared from 24h growth on bovine blood agar (Cottral, 1978). Motility was determined by examination of wet mount preparations using phase-contrast microscopy. Wet mounts

Table 1. *Pasteurella caballi* sp. nov. strains studied

Strain	Location of Sender	Source	Other clinical information
83851[a]	Washington	uterus	adult Thoroughbred mare
84314	California	lung abscess[b]	adult male, respiratory and CNS signs preceeding death
84513	New Mexico	trachea	1-year-old Appaloosa filly
84532	South Carolina	uterus	12-year-old Quarter Horse mare
84678	Indiana	pleural fluid[b]	13-year-old Quarter Horse mare, pneumonia
84687	Washington	uterus	5-year-old mare, metritis
85256	Kentucky	gutteral pouch	10-year-old mare, sinus infection
85683	Maryland	cervix	19-year-old mare, infertile

[a]T, Type strain.

[b]Case histories indicated that *P. caballi* was isolated in high numbers and/or pure culture from these tissues.

Table 1. (continued)

Strain	Location of Sender	Source	Other clinical information
86026	Pennsylvania	bone	2-year-old male, *Actinomyces* sp. also isolated
86200	Montana	wound	4-year-old mare
86514	Wisconsin	lung[b]	4-year-old mare, pneumonia and meningitis
87127	Illinois	lung[b]	14-year-old mare, inhalation pneumonia
87151	California	lung[b]	4-year-old male, respiratory disease, CNS signs
87658	Alabama	lung[b]	3-month-old Quarter Horse filly, fibrinous pneumonia, pleuritis
87659	Alabama	lung	2-month-old Thoroughbred colt, pneumonia, *Rhodococcus equi* also isolated

Table 1. (continued)

Strain	Location of Sender	Source	Other clinical information
88036	Michigan	trachea	5-month-old Thoroughbred colt, pneumonia, *Actinobacillus equuli* also isolated
88037	Michigan	mesenteric abscess[b]	11-year-old male Quarter Horse
88046	Alabama	infected incision	7-year-old Quarter Horse mare
88176	Kentucky	fistulous withers	2-year-old male Thoroughbred, *Streptococcus zooepidemicus* also isolated
88180	Michigan	trachea	5-month-old Standardbred colt, *Bordetella bronchiseptica* also isolated
88181	Michigan	lung	6-month-old Quarter Horse filly
88193	Maryland	brain	6-year-old mare

Table 1. (continued)

Strain	Location of Sender	Source	Other clinical information
88200	Kentucky	spleen	5-year-old male
88228	Kentucky	lung	Adult Standardbred mare, necrotizing pneumonia, *Streptococcus zooepidemicus* and *Pseudomonas aeruginosa* also isolated
88282	Michigan	lung	15-year-old Morgan mare, CNS signs, *Actinobacillus equuli* also isolated
88283	Michigan	growth on back	6-year-old Standardbred male, mixed culture
88322	Michigan	uterus	Adult Standardbred mare, *Streptococcus zooepidemicus* also isolated
88405	Kentucky	peritoneal fluid[b]	11-year-old Thoroughbred mare, died of peritonitis following colic surgery
88406	Kentucky	gutteral pouch abscess[b]	15-year-old pony mare, died after 2 week illness

Table 2. Strains used in DNA relatedness studies

Organism[a]	Other designations	Source	Sender[b]
Pasteurella multocida subsp. *multocida*, NCTC 10322^T	ATCC 43137^T	swine	NCTC
P. multocida subsp. *gallicida*, NCTC 10204^T		bovine	NCTC
P. multocida subsp. *septica*, NCTC 11619		human	NCTC
P. multocida, ATCC 27883^T		swine	ATCC
P. aerogenes, ATCC 27882^T		swine	ATCC
P. anatis, NCTC 11413^T		duck	NCTC
P. avium, ATCC 29546^T		chicken	ATCC
P. canis, NCTC 11621^T		dog	NCTC
P. dagmatis, NCTC 11617^T		human	NCTC

[a]Strain designations are those of the sender. T, type strain.

[b]NCTC, National Collection of Type Cultures, London, England; ATCC, American Type Culture Collection, Rockville, Md. R.E. Weaver, Centers for Disease Control, Atlanta, Ga.; K. Rhoades, National Animal Disease Center, Ames, Ia.; R.F. Ross, Iowa State University, Ames, Ia.; M.L. Kaeberle, Iowa State University, Ames, Ia.; Va., state of origin of culture submitted to the National Veterinary Services Laboratories, Ames.

Table 2. (continued)

Organism	Other designations	Source	Sender
P. gallinarum, P-913	ATCC 13361^T	chicken	Rhoades
P. haemolytica, ATCC 33396^T	NCTC 9380^T	sheep	ATCC
P. langaa, NCTC 11411^T		chicken	NCTC
P. pneumotropica, ATCC 35149^T	NCTC 8141^T	mouse	ATCC
P. stomatis, NCTC 11623^T		dog	NCTC
P. testudinus, ATCC 33688^T		tortoise	ATCC
Actinobacillus equuli, ATCC 19392^T	NCTC 8529	horse	ATCC
A. lignieresii, ATCC 19393^T	NCTC 4976	bovine	ATCC
A. suis, NVSL 88441		horse	Va.
A. ureae, NCTC 10219^T	ATCC 25976	human	NCTC
A. rossii, Ross 63	ATCC 27073	swine	Ross
Haemophilus influenzae, ATCC 33391^T	NCTC 8143	human	ATCC
H. aphrophilus, F3436		human	Weaver
"*H. somnus*" ISU 8025		bovine	Kaeberle

were prepared using growth from chocolate agar (Columbia base; Difco) slants that had been incubated 24h at 25 and 37°C.

Biochemical Tests

Dehydrated media from commercial sources were used whenever possible. Inoculated media for all tests were incubated aerobically at 37°C. Catalase, indophenol oxidase, phosphatase, and glucosidase enzyme test results were recorded at 24h. Tests for nitrate and nitrite reduction and indole production were performed after 48h incubation. The Voges-Proskauer and methyl red tests were read after 4 days incubation. All other biochemical tests were observed daily for one week, and results were considered negative if no reaction was observed by that time. Unless otherwise indicated, methods described by Lányi were used for the conventional biochemical tests (Lányi, 1987). The test for indole production was done using Ehrlich's reagent following extraction with xylene. Indophenol oxidase was detected on filter paper using the tetramethyl-p-phenylenediamine dihydrochloride reagent (Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.). Porphyrins were detected with a commercial disc reagent (Remel, Lenexa, Kans.). Stab cultures for detection of gelatin hydrolysis were incubated at 37°C, then chilled at 4°C for 10 min before results were recorded (Clark *et*

al., 1984). Hydrolysis of esculin was tested on heart infusion agar slants containing 1% (wt/vol) esculin and 0.5% ferric chloride (Phillips and Nash, 1985). The test for methylene blue milk reduction was performed as described by MacFaddin (1980). Glycosidase enzyme tests were performed as described by Kilian and Bulow (1976) using 0.1% (wt/vol) solutions of 2-nitrophenyl-ß-D-galactopyranoside (Calbiochem-Behring, La Jolla, Calif.), 4-nitrophenyl-ß-D-glucopyranosiduronic acid (Sigma Chemical Co., St. Louis, Mo.), and 4-nitrophenyl-α-L-fucopyranoside (Sigma). For fermentation tests, carbohydrates (1%, wt/vol) were incorporated into heart infusion broth (Difco) with 1.0% (vol/vol) Andrade's indicator. Gas production from D-glucose was detected in a Durham tube. Cultures were observed daily for one week for acid and gas production.

DNA Relatedness

Procedures used for the preparation of unlabeled DNA, the hydroxyapatite method for DNA hybridization, and the calculations used to determine relatedness have been described (Brenner *et al.*, 1982). DNA from *P. caballi* 83851^T (T=type strain) was labeled with [$32PO_4$]-dCTP by nick translation using a commercial kit (catalog number 8160SB, Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

Hybridization reactions were done at 55°C (optimal reassociation) and 70°C (stringent reassociation).

Determination of DNA Base Composition

The G+C content of DNA prepared from 4 *P. caballi* strains was determined by denaturing DNA dissolved in SSC (0.15M NaCl plus 0.015M sodium citrate) in a model 2600 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a model 2527 thermoprogrammer. The G+C contents were calculated from the thermal denaturation midpoint temperatures (T_m) using the formula %G+C=(T_m-69.3)/0.41 (Marmur and Doty, 1962). *Proteus mirabilis*, ATCC 14273, was used as a control.

Determination of Cellular Fatty Acids

Cellular fatty acid content of the *P. caballi* and reference strains of *Pasteurellaceae* was determined by gas-liquid chromatography using methods described by Moss and coworkers (1988). Cells for fatty acid analysis were obtained after growth on heart infusion agar with 5% rabbit blood. Fatty acids (as methyl esters) were analyzed by gas-liquid chromatography using the HP 5898 A microbial identification system (Hewlett-Packard, Inc., Avondale, Pa.). Identification of individual fatty acids were

confirmed by hydrogenation, acetylation, and mass spectrometry.

Antimicrobial Susceptibility Tests

Antibiograms were determined by the disk diffusion method (Bauer *et al.*, 1966) using Mueller-Hinton agar (Difco) supplemented with 5% sheep blood and 1% IsoVitaleX (BBL Microbiological Systems, Cockeysville, Md.). The antimicrobials and the concentrations used are listed in Table 3. Zone sizes were measured and interpreted as resistant, intermediate, and susceptible according to the National Committee for Clinical Laboratory Standards (1975).

TABLE 3. Antimicrobial susceptibility results for 29 *P. caballi* strains by agar diffusion

Antimicrobial agent (disk concen.[a])	No. of strains that were:		
	Resistant	Intermediate	Susceptible
Amikacin (30)	0	3	26
Ampicillin (10)	0	0	29
Carbenicillin (100)	0	0	29
Cephalothin (30)	0	0	29
Chloramphenical (30)	0	0	29
Erythromycin (15)	0	3	26
Gentamicin (10)	0	0	29
Kanamycin (30)	0	2	27
Lincomycin (2)	29	0	0
Nalidixic Acid (30)	0	1	28
Neomycin (30)	4	11	14
Penicillin G (10U)	0	8	21
Streptomycin (10)	12	6	11
Sulfadiazine (300)	26	3	0
Tetracycline (30)	0	0	29
Tobramycin (10)	0	0	29
Trimethoprim (5)	11	0	18

[a]All disk concentrations except for Penicillin G are in micrograms.

RESULTS AND DISCUSSION

Sources and Habitat

The sources of the *P. caballi* strains studied and available clinical information are given in Table 1. All of the strains were isolated from equine clinical specimens and were obtained from horses in 13 states. The largest number of strains which were submitted from Kentucky and Michigan may reflect an interest on the part of colleagues rather than an unusually high incidence of this organism in these two states. Horses of all ages were represented. Twenty-four strains were from adult horses aged 1 to 19 years and five were from foals less than 7 months old. Fifteen of the 29 strains were isolated from respiratory sources, suggesting that *P. caballi*, like other pasteurellae, is an inhabitant of the mucous membranes of the pharynx and upper respiratory tract. The *P. caballi* strains were isolated in high numbers and pure culture from horses with upper respiratory infection, pneumonia, peritonitis, and from a mesenteric abscess. This evidence suggests that this organism played a significant role in the pathogenesis of those infections. The clinical significance is less certain in instances where the organism was present in mixed cultures.

Phenotypic Characteristics

The 29 *P. caballi* strains studied were small, nonmotile, bipolar stained, gram-negative rods that gave positive reactions in tests for oxidase, phosphatase, and nitrate reduction. In addition, all strains were facultative anaerobes and all produced acid from fermentation of carbohydrates. Additional characteristics that were common to all strains are given below in the species description. Variable characteristics for the 29 *P. caballi* strains are given in Table 4. The 29 equine strains studied had the characteristics of the family

TABLE 4. Variable phenotypic characteristics of the *P. caballi* strains studied

Test	Cumulative % of strains positive on day: 1	2	7	Reaction of type strain 83851
Acid from:				
myo-inositol	14	14	14	-[a]
lactose	0	14	100	(+)
maltose	76	83	93	+
D-mannitol	93	97	97	+
raffinose	0	7	72	(+)
L-rhamnose	21	21	21	-
D-sorbitol	14	14	14	-
D-xylose	59	59	59	+
Growth at 42°C	93	93	93	+
Ornithine decarboxylase	41	59	66	+
Yellow pigment	76	76	76	+

[a]-, Negative after 7 days; +, positive within 2 days; (+), positive in 3 to 7 days.

Pasteurellaceae (Mannheim, 1984). Of the three genera in this family (i.e., *Pasteurella, Haemophilus, Actinobacillus*), these equine strains are best placed in the genus *Pasteurella* (Carter, 1984; Mutters *et al.*, 1985). Lack of a requirement for preformed growth factors would exclude these strains from the genus *Haemophilus*. Members of the genus *Actinobacillus* typically produce sticky colonies, grow well on MacConkey agar, and hydrolyze urea and esculin, characteristics absent in these strains. Several characteristics unusual for *Pasteurella* were observed: a negative catalase test, production of gas from D-glucose, and the production of acid from *myo*-inositol and L-rhamnose by a minority of strains. However, we do not believe these differences warrant exclusion of these strains from the genus when the numerous morphological and biochemical characteristics in common with *Pasteurella* are considered. Production of gas from D-glucose serves to distinguish *P. caballi* from most other species of *Pasteurellaceae*. The tests that are useful for differentiating *P. caballi* from selected aerogenic *Pasteurellaceae* are given in Table 5. Negative tests for catalase and urease as well as lack of growth on MacConkey agar distinguish *P. caballi* from *P. aerogenes* and *Actinobacillus rossii*. *P. caballi* can be distinguished from *P. dagmatis* by its negative reactions for catalase, indole,

Table 5. Differentiation of *P. caballi* from selected aerogenic species of *Pasteurellaceae*[a]

	Pasteurella caballi	*Pasteurella aerogenes*	*Pasteurella dagmatis*	*Haemophilus aphrophilus*	*Actinobacillus rossii*
MacConkey	-	+	-	-	+
Catalase	-	+	+	-	+
CO_2 enhances growth	-	-	-	+	-
Indole	-	-	+	-	-
ß-hemolysis	-	-	-	-	+
Ornithine decarboxylase	d[b]	d	-	-	-
Urease	-	+	+	-	+
Acid from:					
L-arabinose	-	d	-	-	+
myo-inositol	d	d	-	d	+
L-rhamnose	d	d	-	-	+
D-sorbitol	d	d	-	-	+
Trehalose	-	-	+	+	-
D-xylose	d	d	-	-	+
Usual host	horse	pig	dog, cat	human	pig

[a]Data for previously described species adapted from Carter, 1980; Kilian and Biberstein, 1980; Kilian and Fredericksen, 1981; and Mutters *et al*, 1985.

[b]d, Strains vary within species.

urease, and fermentation of trehalose. *P. caballi* is phenotypically most similar to *Haemophilus aphrophilus*, but can be differentiated from that organism by its negative reaction for trehalose fermentation and its ability to grow well without additional carbon dioxide. The source of an isolate would be of some importance in the initial differentiation of *P. caballi* from *H. aphrophilus*, since each appears to have a limited host range.

Antimicrobial Susceptibility

The responses of *P. caballi* to 17 antimicrobial agents are shown in Table 3. The majority of the strains were resistant to lincomycin and sulfadiazine. Resistance to streptomycin, neomycin, and trimethoprim was also observed in some strains. These results are generally consistent with those reported for other members of *Pasteurella* (Fales *et al.*, 1982).

Fatty Acid Analyses

The fatty acid composition of the *P. caballi* strains was similar to that of strains of 12 other species of *Pasteurellaceae* examined (Table 6). Relatively large amounts of tetradecanoic (14:0), hexadecenoic (16:1 ω7c; palmitoleic), and hexadecanoic (16:0; palmitic) acids were found. Also present, but in lower concentrations, were

Table 6. Cellular fatty acid composition of *P. caballi* and selected species of *Pasteurellaceae*

Strains	No. of strains	Fatty acids (% of total)						
		14:0[a]	3-OH 14:0	16:1 ω7c	16:0	18:2	18:1 ω9c	18:0
P. caballi	29	30(2)[b]	9(2)	28(3)	16(2)	5(1)	4(1)	2(1)
P. multocida	11	19(3)	5(1)	29(2)	32(3)	4(1)	2(1)	3(1)
P. dagmatis	4	25(3)	8(1)	29(5)	22(2)	6(2)	3(1)	3(1)
P. pneumotropica	3	25(5)	5(1)	31	31(1)	1	2(1)	2(2)
P. aerogenes	2	22	7(1)	31(2)	28	3	2	2
P. canis	2	21(7)	6	24(5)	28(8)	9	4(1)	4(3)

[a]Numbers before the colon indicate the number of carbon atoms and numbers after the colon refer to the number of double bonds. 3-OH indicates hydroxy group at carbon 3; ω7c and ω9c indicate double bond position from the hydrocarbon end of the carbon chain; c is cis isomer.

[b]The first number is the arithmetic mean of the relative percent of the fatty acid; (n) is the standard deviation; absence of (n) indicates the standard deviation was less than 0.6%. All values were rounded to the nearest whole number. T, less than 0.6% detected.

Table 6. (continued)

Strains	No. of strains	Fatty acids (% of total)						
		14:0[a]	3-OH 14:0	16:1 ω7c	16:0	18:2	18:1 ω9c	18:0
P. stomatis	2	25(4)	10(3)	22(5)	21(3)	9(1)	6(1)	3
A. equuli	4	25(5)	7(1)	27(4)	19(2)	8(3)	5(2)	3(2)
A. lignieresii	3	25(2)	7(1)	31(2)	22(2)	5(1)	3	2
A. ureae	3	29(5)	6	23(5)	24(5)	6(2)	5(2)	3(1)
A. rossi	2	32(6)	7(1)	32	18(5)	4(1)	2	1
H. aphrophilus	7	21(4)	6(2)	26(9)	33(3)	5(3)	4(2)	2(1)
H. influenzae	4	18(6)	4(2)	35(3)	35(3)	1	T	2(1)

3-hydroxy-tetradecanoic (3-OH-14:0), cis-9,12 octadecanoic (18:2; linoleic), cis-9-octadecenoic (18:1 9ωc; oleic), and octadecanoic (18:0; stearic) acid. A small amount (2% or less) of dodecanoic (12:0; lauric) acid and an isomer of tetradecenoic (14:1) were also present in all strains examined. These results are in general agreement with those of Jantzen and coworkers (1981) who examined 18 species of *Pasteurellaceae*, except that we obtained smaller amounts of 3-OH-14:0 acid. This is due to differences in methods, because our base hydrolysis procedure does not quantitatively liberate all amide-linked hydroxy acids. Although fatty acid composition is of no value in differentiating among genera and species of *Pasteurellaceae*, all members of the family are easily distinguished from other small, gram-negative, rod-shaped bacteria by their characteristic fatty acid composition.

DNA Studies

The mean G+C content of DNA from 4 *P. caballi* strains, including the type strain, was 41.2 to 42.4 mol%. These values are within the range (37.7 to 45.9) given for the genus *Pasteurella* (Carter, 1984). The results of DNA-DNA hybridization experiments are shown in Table 7. Strains

Table 7. DNA relatedness of *Pasteurella caballi* strains

Source of unlabeled DNA	Relatedness to $^{32}PO_4$-labeled DNA from strain 83851^T		
	RBR[a] at 55°C	%D[b]	RBR at 70°C
P. caballi 83851	100	0.0	100
P. caballi 85683	99	1.0	85
P. caballi 88176	98	1.0	77
P. caballi 84679	97	1.0	86
P. caballi 88282	97	1.0	88
P. caballi 84513	94	1.0	91
P. caballi 88228	94	2.0	89
P. caballi 87127	94	1.5	87
P. caballi 84532	93	1.0	86
P. caballi 85256	93	1.0	77
P. caballi 87658	93	1.5	82
P. caballi 88283	93	1.0	85
P. caballi 88405	93	1.5	86
P. caballi 86514	92	1.0	87
P. caballi 88200	92	1.0	87
P. caballi 87151	91	1.5	85
P. caballi 86026	90	1.0	88
P. caballi 88180	90	1.0	85
P. caballi 84314	89	1.0	85
P. caballi 84687	89	1.0	85
P. caballi 87659	89	1.0	84
P. caballi 88193	89	1.5	89
P. caballi 88322	89	1.0	84
P. caballi 88046	88	1.0	87
P. caballi 86200	87	1.0	82
P. caballi 88036	87	1.0	85
P. caballi 88181	87	1.0	88
P. caballi 88037	85	1.0	82
P. caballi 88406	83	1.0	82

[a]RBR, Relative binding ratio = percent of DNA bound to hydroxyapatite in heterologous reactions / percent DNA bound to hydroxyapatite in homologous reactions X 100.

[b]%D, Percent divergence was calculated on the assumption that 1% decrease in thermal stability of a heterologous DNA duplex was caused by 1% unpaired bases.

Table 7. (continued)

Source of unlabeled DNA	Relatedness to $^{32}PO_4$-labeled DNA from strain 83851^T		
	RBR[a] at 55°C	%D[b]	RBR at 70°C
Pasteurella langaa NCTC 11411^T	53	11.5	21
Actinobacillus rossii ATCC 27073	36	14.0	11
Pasteurella pneumotropica ATCC 35149^T	34	5.0	32
Pasteurella multocida subsp. *gallicida* NCTC 10204^T	33	10.5	22
Pasteurella canis NCTC 11621^T	28	12.0	12
Pasteurella multocida subsp. *septica* NCTC 11619	27	14.0	12
Pasteurella multocida subsp. *multocida* NCTC 10322^T	26	15.5	9
Pasteurella avium ATCC 29546^T	25	15.0	7
Pasteurella dagmatis NCTC 11617^T	25	16.5	7
Actinobacillus equuli ATCC 19396^T	24	15.5	10
Actinobacillus lignieresii ATCC 19393^T	22	7.0	19
Pasteurella anatis NCTC 11413^T	21	17.0	4

Table 7. (continued)

Source of unlabeled DNA	Relatedness to $^{32}PO_4$-labeled DNA from strain 83851^T		
	RBR[a] at 55°C	%D[b]	RBR at 70°C
Pasteurella stomatis NCTC 11623^T	21	16.5	5
Pasteurella testudinis ATCC 33688^T	20	13.0	12
Actinobacillus ureae ATTC 10219^T	20	13.0	13
Haemophilus aphrophilus F3436	20	16.5	6
Haemophilus influenzae ATCC 33391^T	22	17.0	5
"Haemophilus somnus" ISU 8025	20	15.0	15
Pasteurella gallinarum P-913	19	18.0	7
Actinobacillus suis NVSL 88441	18	16.0	6
Pasteurella aerogenes ATCC 27882^T	14	16.5	9
Pasteurella haemolytica ATCC 33396^T	13	15.5	8

designated *P. caballi* formed a single DNA relatedness group. The levels of relatedness of labeled *P. caballi* DNA to DNA from 28 other *P. caballi* strains were 83 to 99% (average 91%) in reactions at the optimal reassociation temperature. Divergence in related DNA sequences was 1 to 2%. Relatedness remained high (average 85%) under stringent reassociation conditions. Strain 83851^T was 53% or less related to other species of *Pasteurellaceae*, indicating that the equine strains represent a species not previously described. Closest relatedness occurred with *Pasteurella langaa*. Relatedness to other *Pasteurellaceae* was 13 to 34%.

In summary, results of phenotypic characterization, fatty acid analyses, and DNA-DNA hybridization studies support the conclusion that these equine strains constitute a new, homogenous taxon in the genus *Pasteurella*. It is proposed that these strains be designated *Pasteurella caballi* sp. nov.

Description of *Pasteurella caballi* sp. nov.

Pasteurella caballi (ca.bal'li. L.n. *caballus* a horse; L.gen.n. *caballi* of a horse; *Pasteurella caballi* the *Pasteurella* of a horse) cells are rod-shaped (0.8 to 1.0 μm by 1.3 to 1.9 μm), bipolar stained, and arranged singly or in pairs with occasional swollen, curved, or filamentous forms observed. Gram-negative. Not acid-fast. Endospores

are not formed. Nonmotile. Facultatively anaerobic. Colonies are 1.0 to 1.5 mm in diameter, smooth, slightly raised, grayish-yellow on blood agar after 24h incubation. Growth at room temperature and at 37°C, but not at 4°C. Colonies are not hemolytic on bovine, sheep, or rabbit blood agar, but do impart a brownish discoloration to these media under areas of confluent growth. Growth scraped from the surface of the agar is cream (less frequently) to bright yellow (usual) in color. Growth on MacConkey agar is scant or absent. X factor and V factor independent. Nitrate is reduced to nitrite. Positive for oxidase, phosphatase, and ß-galactosidase. Methylene blue milk is reduced. Porphyrins are produced from δ-aminolevulinic acid. Catalase, indole, lysine decarboxylase, arginine dihydrolase, α-fucosidase, and ß-glucuronidase are not produced. Esculin, gelatin, and urea are not hydrolyzed. Citrate is not utilized. Voges-Proskauer and methyl red are negative. D-glucose is fermented with the production of acid and gas. Acid is produced from D-fructose, D-galactose, lactose, D-mannose, and sucrose. Fermentation of lactose is delayed. Acid is not produced from adonitol, L-arabinose, cellobiose, dulcitol, inulin, salicin, starch, or trehalose. Most strains are sensitive to amikacin, ampicillin, carbenicillin, cephalothin, chloramphenicol, erythromycin, gentamicin, kanamycin, nalidixic acid,

tetracycline, and tobramycin. Contains 3-OH-14:0 and approximately 25% each of 14:0, 16:1 ω7c, and 16:0 acids, which is the characteristic fatty acid profile of all *Pasteurellaceae*. The G+C content of DNA is 41 to 42 mol% (T_m). *Pasteurella caballi* strains have been isolated from equine clinical specimens. The type strain of *P. caballi* is 83851 (=ATCC 49197), and it exhibits all of the characteristics described above. Additional variable characteristics are given in Table 4. The type strain was isolated in 1983 from the uterus of a mare. The G+C content of the DNA was 42 mol%.

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GENERAL SUMMARY

The objective of this study was to confirm that the "aerogenic *Pasteurella*-like organisms from horses" constitute a new species. The evidence from DNA-DNA hybridization analyses support this conclusion, and we have proposed the name *Pasteurella caballi* for this new species. A genetic species has been defined as a group of strains that are 70% or more related at optimal conditions and 55% or more related at less than optimal (stringent) conditions and whose DNAs contain 6% or less divergence in related sequences (Brenner, 1981). Average relatednes of *P. caballi* DNAs was 91% (optimal conditions) and 85% (stringent conditions). Divergence in related sequences was 1 to 2%.

After it was established that *P. caballi* was a genetic species, we searched for characteristics that would allow the veterinary microbiologist to distinguish *P. caballi* from phenotypically similar *Pasteurellaceae*. It is of limited use to describe a new species if it cannot be identified phenotypically with routine biochemical tests. *P. caballi* is not difficult to identify. Production of gas from D-glucose distinguishes it from most other *Pasteurellaceae*. Tests for production of catalase, indole, ornithine decarboxylase, urease, and fermentation of trehalose may be used to separate *P. caballi* from other aerogenic *Pasteurellaceae*.

The question of genus assignment was more difficult. Members of the same genus should be more closely related to each other than to species in other genera (Farmer *et al.*, 1980). Our studies revealed considerable overlap in percent relatedness between the type strain of *P. caballi* and members of the three genera in *Pasteurellaceae*. Strains of *P. caballi* were 19 to 53% related to members of *Pasteurella* sensu stricto and 13 to 36% related to species of other genera. (*P. aerogenes* and *P. haemolytica* were considered as species of other genera even though they still have the name *Pasteurella*.) *P. caballi* was more closely related to the type species of *Pasteurella* (26-33%) than to *Actinobacillus lignieresii* (22%) or to *Haemophilus influenzae* (22%). On the basis of the closer genetic relationship by DNA-DNA hybridization and phenotypic similarity to members of the genus *Pasteurella*, we believe *Pasteurella* is the most logical assignment for the equine strains.

The name *Pasteurella caballi* was chosen for this new species because all of the strains had been isolated from horses and we wanted to convey the message that this is "the *Pasteurella* of the horse". We realize that choosing a name based on host may be somewhat hazardous. (It may even ensure that our next isolate is from another animal.) However, with the exception of *P. multocida*, the

pasteurellae are associated with one or two preferred hosts, and we are confident this name is appropriate.

As a consequence of this description, we sincerely believe *P. caballi* will be recognized by veterinary microbiologists when it is encountered in equine clinical specimens. Increased recognition will allow us all to learn more about the prevalence of this organism and its association with disease conditions in the horse.

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