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Taxonomic Subgroups of *Pasteurella multocida* Correlate with Clinical Presentation

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Pasteurella multocida **is a small nonmotile gram-negative coccobacillus that is found in the nasopharynx and gastrointestinal tract of many wild and domesticated animals. In humans it most commonly causes cellulitis and localized superficial skin abscesses following an animal bite or scratch. The respiratory tract is the second most common site of infection for *Pasteurella*. Of the more than 17 species of *Pasteurella* known, *Pasteurella multocida* subsp. *multocida* and *Pasteurella multocida* subsp. *septica* are among the most common pathogens in humans. With the use of molecular techniques, distinction between different subspecies of *P. multocida* can be made more easily and accurately. We used the sequence of the 16S ribosomal DNA (rDNA) and repetitive extragenic palindromic sequence-PCR (REP-PCR) to characterize 20 strains (14 of *P. multocida* subsp. *multocida* and 6 of *P. multocida* subsp. *septica*; the 16S rDNA is identical for *P. multocida* subsp. *multocida* and *Pasteurella multocida* subsp. *gallicida* but differs from that of *P. multocida* subsp. *septica*) isolated from various anatomic sites. We found excellent correlation between the 16S rDNA sequence (a marker for a small conserved region of the genome), REP-PCR (a marker for a large portion of the genome), and biochemical tests (trehalose and sorbitol). We also found a correlation between the clinical presentation and the taxonomic group, with *P. multocida* subsp. *septica* more often associated with wounds than with respiratory infections (67 versus 17%, respectively) ($P < 0.05$, Z test) and *P. multocida* subsp. *multocida* more often associated with respiratory infections than with wounds (71 versus 14%, respectively) ($P < 0.05$, Z test).**

Pasteurella multocida is a small nonmotile gram-negative coccobacillus that is found in the nasopharynx and gastrointestinal tract of many wild and domesticated animals. It does not often result in disease in its animal hosts. However, in humans it most commonly causes cellulitis and localized superficial skin abscesses following an animal bite or scratch (5, 12).

The respiratory tract is the second most common site of infection for *Pasteurella*, where it may present as pneumonia, tracheobronchitis, abscess, or empyema. The organism may also cause upper respiratory infections such as sinusitis and pharyngitis (7). Of these, pneumonia is the most common manifestation of respiratory infection caused by *Pasteurella*, and patients may acutely or insidiously present with fever, dyspnea, and pleuritis. Those who develop respiratory infection from *Pasteurella* also tend to be elderly and have underlying chronic lower respiratory tract disease, and the route of infection appears to be through inhalation. The organism may also be opportunistic and affect immunocompromised patients as well, causing pneumonia in patients with AIDS and immunoglobulin A deficiency (4, 6). Much less frequently, *Pasteurella* causes osteomyelitis, intra-abdominal infections, septic arthritis, sepsis, and meningitis (1, 12).

Of the more than 17 species of *Pasteurella* known, *Pasteurella multocida* subsp. *multocida*, *Pasteurella multocida* subsp. *septica*, *Pasteurella canis*, *Pasteurella stomatis*, and *Pasteurella dogmatis* are the most common pathogens in humans (8). Cats have the highest rate of oropharyngeal colonization by *P. multocida* (50 to 90%), followed by dogs (50 to 66%), pigs (51%), and rats (14%) (12). This distribution is reflected by the greater chance of isolating *Pasteurella* from cat bites (50%) than from dog bites (20 to 30%), although dog bites are much more common (5). In most cases of disease, the organism has been acquired either directly through bites or aerosol inhalation or indirectly by contact with fomites contaminated with animal secretions. Interestingly, *Pasteurella* sp. may also become part of the normal respiratory tract flora in humans. It has been found in healthy veterinary students and animal handlers without any pulmonary symptoms (10).

As for other organisms, with the use of molecular techniques, distinction between different subspecies of *P. multocida* can be made more easily and accurately (3). One can detect whether there are any genotypic or subspecies differences between the organisms that cause skin infections and those that cause respiratory infections. Sequencing of the 16S ribosomal DNA (rDNA) and repetitive extragenic palindromic sequence-PCR (REP-PCR) have been used previously to distinguish strains of *Francisella*, *Bartonella*, and *Mycobacterium* (2, 3; T. Raich and J. E. Clarridge, Abstr. 95th Gen. Meet. Am. Soc. Microbiol. 1995, abstr. U-99, p. 134, 1995). We used these methods to characterize strains of *Pasteurella* isolated from various human sites.

MATERIALS AND METHODS

Identification of strains and correlation with clinical presentation. The 20 *P. multocida* isolates were determined from the examining microbiology records for the years between 1985 and 2002 at the Veterans Administration Medical Center in Houston, Tex. These strains were identified originally by conventional microbiological and biochemical methods including Gram stain, colony morphology, oxidase reaction, and RapID NH identification kits (Remel Inc., Lenexa, Kans.).

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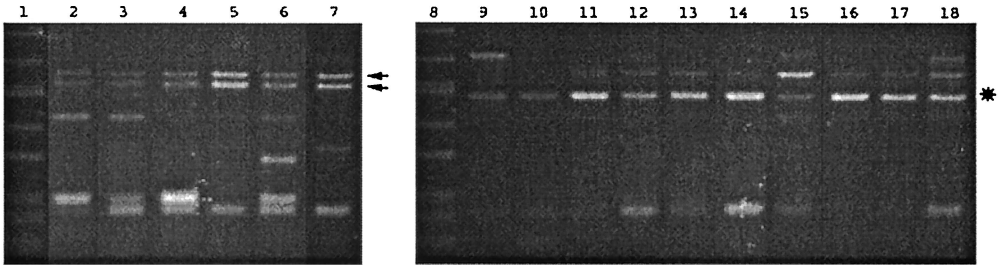

FIG. 1. Gel of REP-PCR products from various clinical isolates. Lanes 1 and 8 represent molecular size markers (Sigma, St. Louis, Mo.). Lanes 2 to 7 (pattern A) were confirmed as *P. multocida* subsp. *septica* by 16S rDNA sequence. In contrast lanes 9 to 18 (pattern B) were demonstrated to be *P. multocida* subsp. *multocida*. The double bands marked by arrows are between approximately 1,600 and 2,000 bp in size. The asterisk denotes the band migrating slightly below the 1,400-bp marker.

At the time of identification, a stock of each organism was frozen at −70°C with glycerol. For this study, the organisms were subcultured on chocolate agar in 8% CO_2 for 24 to 36 h. Additional biochemical tests were performed using the API 20E and Rapid ID 32 Strep kits (Biomerieux, Lyon, France). Patients' histories were reviewed from computer records when available. Statistical significance was determined by the Z test.

REP-PCR. The organisms were harvested and resuspended in 0.9% sterile saline to a 3.0 McFarland standard turbidity. The PCR primers REP1R and REP2-1 as well as previously described methods using conserved primers to REP sequences in PCR were used (2, 11; Raich and Clarridge, Abstr. 95th Gen. Meet. Am. Soc. Microbiol. 1995). The reaction mixture consisted of the following: 2 μl of bacterial suspension, 50 mM KCl, 10 mM Tris HCl, 0.1% Triton X-100, 3.0 mM $MgCl_2$, 10% dimethyl sulfoxide, 200 μM (each) deoxynucleoside triphosphates, 100 pmol of each primer, and 1 U of *Taq* polymerase (Promega, Madison, Wis.). Amplification was performed on a Perkin-Elmer GeneAmp 9600 PCR system (Perkin-Elmer, Norwalk, Conn.) as follows: 1 cycle of 95°C for 5 min; 35 cycles of 94°C for 1 min, 40°C for 1 min, and 65°C for 4 min; 1 cycle of 65°C for 10 min; and final holding at 4°C until analysis. The PCR products were separated by electrophoresis on a 1.2% agarose–ethidium bromide gel in 0.5% TBE buffer (0.045 M Tris-borate, 0.001 M EDTA) at 80 V.

16S rDNA sequence analysis. 16S rRNA gene sequence identification was performed using the MicroSeq 500 Gene Kit (Applied Biosystems, Foster City, Calif.) and the 3100 Genetic Analyzer (Hitachi, Tokyo, Japan) according to manufacturer's specifications. Approximately 500 bp in both forward and reverse sense were sequenced for each isolate. Test strain sequences were compared against the MicroSeq 16S rRNA gene sequence database. The database contains sequences from 1,297 different species (1,187 type strains) including type strains from the genus *Pasteurella-Haemophilus-Actinobacillus* groups. Sequence data obtained from GenBank for nontype strains were also included in the analysis. Sequences were compared in dendrogram form by the neighbor-joining method using the MicroSeq statistical package (9).

RESULTS

Analysis of the REP-PCR products revealed the presence of two predominant patterns (Fig. 1) which we termed patterns A and B. Although there are variations in the number of bands present among the different specimens belonging to one particular pattern, each group shares common characteristics. Pattern A is distinguished by a closely separated double band at the 1,600- to 2,000-bp region (arrows in Fig. 1). However, pattern B is recognizable by the common presence of a band migrating slightly below 1,400 bp (asterisk in Fig. 1). The variable presence of some bands may be partially due to differences in the intensity of staining.

The separation of the specimens into two distinct groups correlates with the subspecies classification of each organism by 16S rDNA sequence. By comparing 16S rDNA sequence to known sequences of *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*, one can assign each isolate to either subspecies (Fig. 2). The sequence of the 16S rDNA showed very high homology between *P. multocida* subsp. *septica* and *P. multocida* subsp. *multocida*. By this method, the organisms that showed pattern A by REP-PCR also had a 16S rDNA sequence homologous to that of *P. multocida* subsp. *septica*. In contrast, those with pattern B had the 16S rDNA sequence of *P. multocida* subsp. *multocida*. Of note, *P. multocida* subsp. *multocida* and *Pasteurella multocida* subsp. *gallicida* have identical 16S rDNA sequences.

The subspecies *P. multocida* subsp. *multocid*a and *P. multocida* subsp. *septica* share very similar biochemical profiles. With the identification kits mentioned in Materials and Methods, consistent differences were seen only in the sorbitol and trehalose biochemical tests. In our isolates, *P. multocida* subsp. *multocida* was positive for sorbitol and negative for trehalose metabolism, whereas *P. multocida* subsp. *septica* was variable for sorbitol and positive for trehalose.

We correlated the site and disease process from which each isolate was obtained with the subspecies. Of the 20 strains studied, 14 belonged to *P. multocida* subsp. *multocida* subspecies, and the other 6 were classified as *P. multocida* subsp. *septica* (Table 1). The majority (10 of 11, or 91%) of the strains causing respiratory infections were of *P. multocida* subsp. *multocida*. In addition, there were two nonwound and nonrespiratory cases caused by *P. multocida* subsp. *multocida*: one case of bacteremia and one case of peritoneal cavity seeding.

In contrast, four of the six strains of *P. multocida* subsp. *septica* were isolated from various wound infections, while only one was seen in a case of pneumonia. The last strain of *P. multocida* subsp. *septica* was isolated from the blood. Thus, *P. multocida* subsp. *septica* was more often found in infected wounds than in respiratory sites, but the number was not statistically significant. In summary, the relative proportions of wound and respiratory cases were statistically different between the subspecies ($P = 0.05$, Z test). Of the respiratory cases, *P. multocida* subsp. *multocida* was more likely the cause ($P < 0.05$, Z test). Conversely, *P. multocida* subsp. *multocida* had a greater propensity to cause respiratory rather than wound infections ($P = 0.05$, Z test).

Upon review of patient records, most of the patients diagnosed with *Pasteurella* respiratory infections also had underlying chronic pulmonary disease, including chronic obstructive pulmonary disease and primary lung cancer. These cases often presented with a mild form of pneumonia. Also, most of the

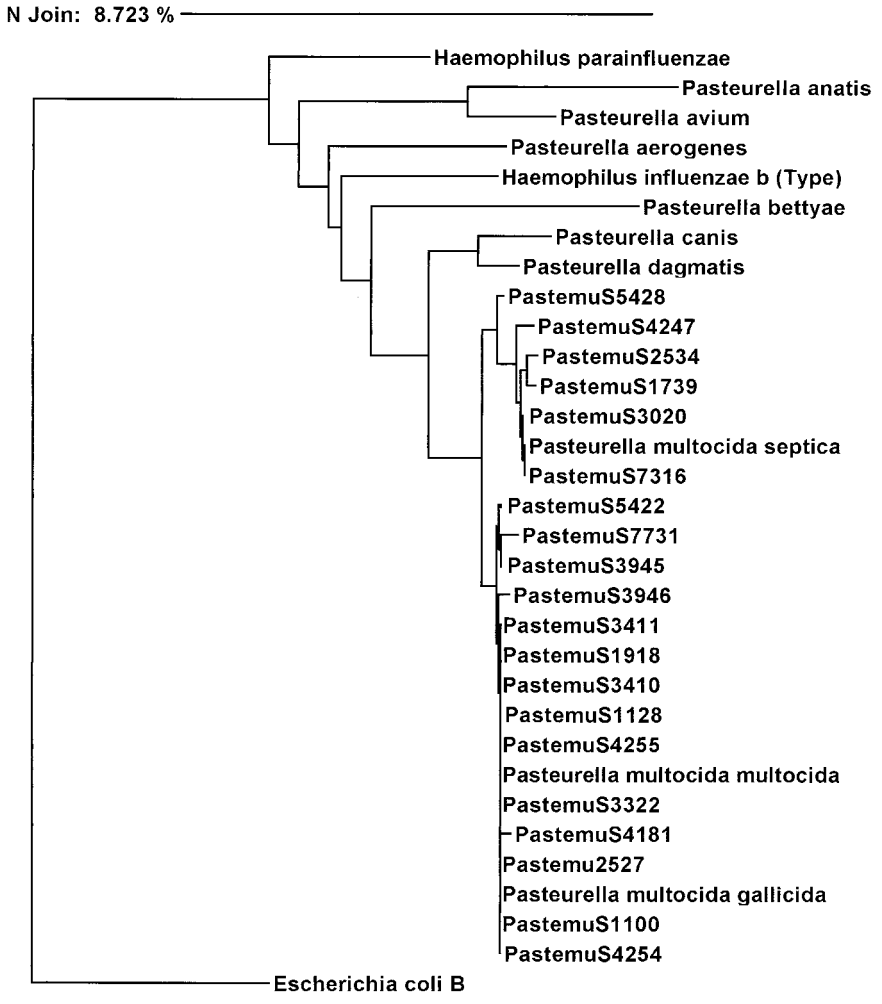

FIG. 2. Neighbor-joining tree of *P. multocida* clinical isolates based on their 16S rDNA sequences. The isolates (those designated with a number) are grouped with either *P. multocida* subsp. *multocida* or *P. multocida* subsp. *septica* based on their 16S rDNA sequence similarity. Type strains are noted by their full genus and species name and are ATCC 43137, ATCC 19427, and ATCC 29977 for *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, and *P. multocida* subsp. *gallicida*, respectively. *Escherichia coli* is the outgroup.

patients with cases of wound infection had prior animal contact, and the cases were characterized by a localized skin infection.

DISCUSSION

By two independent molecular techniques, distinct strain differences were identified between divergent disease processes. In our series, *Pasteurella* respiratory infections most often involved *P. multocida* subsp. *multocida*. Interestingly, *P. multocida* subsp. *multocida* itself seems to have a propensity for respiratory sites and infections. However, *P. multocida* subsp. *septica* causes respiratory symptoms less often and was found more frequently in infected wounds.

TABLE 1. Subspecies distribution of clinical isolates[a]

Subsp.	No. of isolates examined (%) by site:		
	Wound	Respiratory	Other
P. multocida subsp. *multocida*	2 (14)	10 (71)	2 (14)
P. multocida subsp. *septica*	4 (67)	1 (17)	1 (17)

[a] The clinical isolates are grouped into either *P. multocida* subsp. *multocida* or *P. multocida* subsp. *septica* by 16S rDNA sequence and REP-PCR pattern. They are also separated by their respective clinical sites. Numbers and percentages for each clinical site within the subspecies are shown.

The 16S rDNA sequence has been accepted as a reliable barometer of evolutionary change between different bacterial species. It shows distinct differences between subspecies as well, which makes the technique ideal for our purposes. Similarly, in the clinical setting DNA sequencing and not biochemical assays may be the most reliable and efficient method to distinguish between the different subspecies of *Pasteurella*. The various clinically significant subspecies of *Pasteurella* share many similar biochemical properties, and results are often equivocal. In contrast to earlier published reports (1), we found that the metabolism for trehalose is distinctly different between *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*.

Furthermore, the process of studying patterns in the genomic DNA by REP-PCR also appears to be accurate and

sensitive. With it, we were able to distinguish between *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*. Since REP-PCR can detect differences in the entire genomic DNA by virtue of the various locations of the repetitive extragenic sequences, it may be more sensitive than 16S rDNA sequencing in other organisms.

We found excellent correlation between the 16S rDNA sequence (a marker for a small conserved region of the genome), REP-PCR (a marker for a large portion of the genome), and biochemical tests (trehalose and sorbitol). Although the number of the cases is small, we found a statistically significant correlation with the type of disease. Thus, in the case of *Pasteurella*, genotypic and phenotypic differences between the various subspecies may result in divergent patterns of pathogenicity. Further investigation would be useful to clarify these relationships.

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