NOTE *Laboratory Animal Science*

Phylogenetic relationship of *Pasteurella pneumotropica* Isolates from Laboratory Rodents Based on 16S rDNA Sequence

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(Received 29 September 2005/Accepted 14 February 2006)

ABSTRACT. A 1344 bp fragment of the 16S ribosomal DNA (rDNA) sequence was used to determine the genetic relationship of *Pasteurella pneumotropica* isolates from laboratory rodents. A total of 30 nucleotide sequences of *P. pneumotropica*, including 24 wild strains, 3 reference strains, and 3 nucleotide sequences deposited in GenBank, were examined for heterogeneity of their 16S rDNA sequences. Phylogenetic analysis based on 16S rDNA sequence discriminated 5 types of branching lineages. Of these 5 types, 3 types had significant associations with mice or rats, and 2 had significant associations with the β-hemolytic phenotype. These results suggest that 16S rDNA sequencing of *P. pneumotropica* isolates demonstrates genetic heterogeneity and phylogenetic discrimination in terms of their hemolytic phenotype and host associations.

KEY WORDS: *Pasteurella pneumotropica*, Phylogenetic analysis, 16S ribosomal DNA.

J. Vet. Med. Sci. 68(6): 639–641, 2006

Recently, immunodeficient animals have become an important source for experimental medicine. Consequently, managing pathogens that do not significantly affect the health of immunocompetent animals, but cause severe diseases in immunodeficient animals has become an important issue. Among them, *Pasteurella pneumotropica* is an infectious agent that is frequently isolated from the upper respiratory tract, lungs, vagina, and digestive tract of laboratory rodents. *Pasteurella pneumotropica* is generally considered an opportunistic pathogen in rodents [13]. In immunodeficient animals, *P. pneumotropica* infection causes lethal pneumonia. Chapes *et al.* [3] and Harts *et al.* [6] reported that toll-like receptor 4 is indispensable for defense against *P. pneumotropica* infections in mice. However, the virulence factors of *P. pneumotropica* are not yet understood. In taxonomy, *P. pneumotropica* is classified into two biotypes. However, *P. pneumotropica* has not yet been formally classified under the genus *Pasteurella* [4, 13, 15]. Although the biochemical properties of *P. pneumotropic*a have been aligned, some phenotypic (e.g. colony morphology and hemolytic activity) and genetic characteristics show heterogeneity. Furthermore, details regarding the phylogeny of wild type strains of *P. pneumotropica* have not yet been clarified. In the present study, we investigated the phylogenetic relationship of *P. pneumotropica* isolates based on 16S rDNA sequence, and compared them with their associations with mice or rats and their hemolytic phenotypes.

The *Pasteurella pneumotropica* reference strains sequenced in this study were CCUG 26451 (biotype Jawetz), CCUG 998 (biotype Heyl), and CCUG 26453 (biotype Heyl). All reference strains were obtained from the Culture Collection University of Göteborg (Göteborg, Sweden). The isolates from the upper respiratory tract of the healthy laboratory rodents were identified by the methods of Lesher *et al.* [11] with the following minor modifications: positive results for glucose and sucrose utilization, ornithine decarboxylase, urease, nitrate reductase, catalase, oxidase, and β-galactosidase; and negative results for motility, H_2S production, citrate utilization and glucose oxidation. All biochemical characteristics were completely identical among the *P. pneumotropica* isolates. The isolates identified as *P. pneumotropica* were obtained from 17 laboratory animal institutes. The bacterial DNA was extracted by the method of Boom *et al.* [1] and used as the template DNA for PCR. The nucleotide sequences of the primers were as follows: primer 1, 5'-GAGAGTTTGATCCTGGCTCAG-3', and primer 2, 5'-AAGGAGGTGATCCAGCCGCA-3'. The thermal cycle program was run on a model PTC-100 thermal controller (MJ Research, MA, U.S.A.) and consisted of initial denaturation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1.5 min. Finally, a 2 min extension at 72°C was also carried out. The amplified PCR products were purified using a Montage PCR purification kit (Millipore, MA, U.S.A.), and then cycle sequencing was carried out with BigDye Terminator Premix (Applied Biosystems, CA, U.S.A.). The products of the sequencing reaction were analyzed using a model ABI 3730XL DNA analyzer (Applied Biosystems). A 1534 bp fragment was sequenced, and the sequencing accuracy was confirmed by two-directional sequencing. The accurate region (1344 bp) among the isolates was used for phylogenetic analysis since there was an uncertain sequence at both the ends of the sequenced region. Multiple alignment analysis, distance matrix calculation and construction of a phylogenetic tree were carried out using the ClustalW program [18]. A phylogenetic tree was generated using the neighbor-joining algorithm [17] and the Tree View program [16] for the isolates and databases (accession no. AF224296, AF012090, and AY362924) deposited in GenBank.

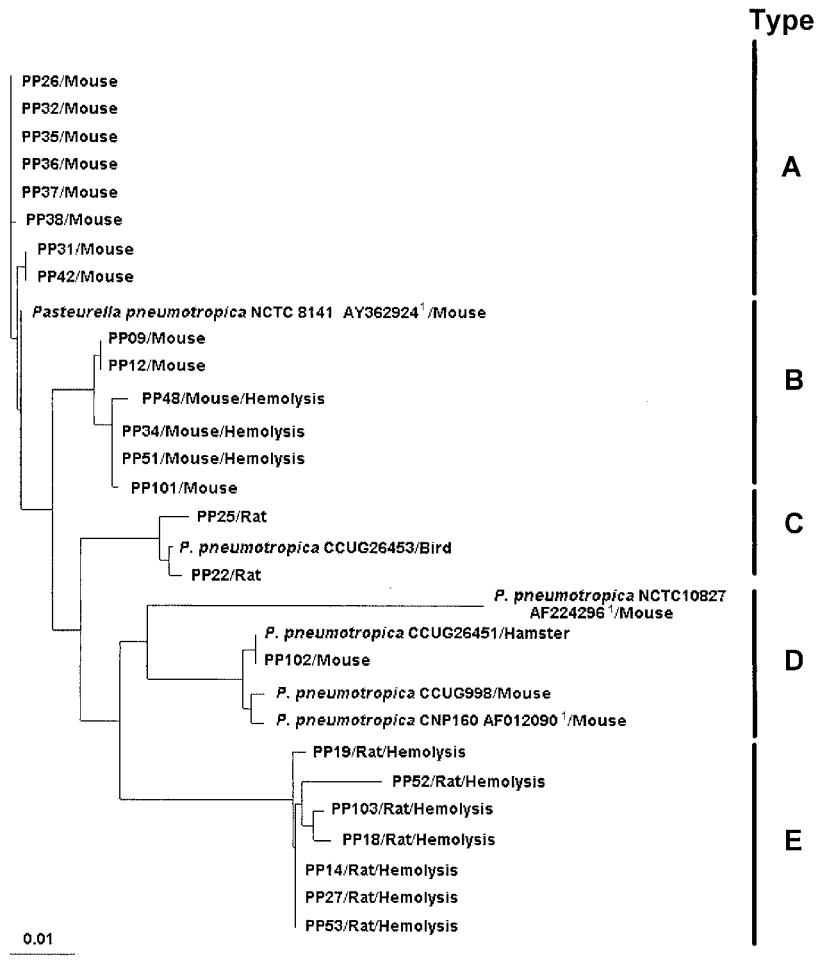

Fig. 1. Phylogenetic analysis of the isolates and reference strains of *P. pneumotropic*a based on the nucleotide sequences of the 16S rDNA gene. The neighbor-joining method was used to construct a phylogenetic tree using the ClustalW program. The strain number, animal source, and hemolytic activity (hemolysis) of the isolates have been represented. 1: Represents the accession no. in GenBank.

The isolates from the upper respiratory tracts of 15 mice and 9 rats were identified as *P. pneumotropica*. Of these, 3 isolates from mice and 7 from rats possessed β-hemolytic activity on blood agar. A phylogenetic tree was generated from 27 strains and 3 nucleotide sequences deposited in GenBank (Fig. 1). The host of origin and β-hemolytic activity (hemolysis) of each strain were also shown. The phylogenetic tree represents 2 major lineages, type A and the other types (B, C, D, and E). The other types were further divided into 4 subtypes by branching lineages. There were no close relationships with regard to the source of laboratory animal institutes among these types. All the strains that belonged to type A were nonhemolytic and derived from mice. All 6 type B isolates were derived from mice, and 3 of these isolates showed β-hemolytic activity on horse blood agar. The type C and D isolates, reference strains, and database sources of were associated with various host animals, including mice, rats, hamsters, and birds. Furthermore, none of these strains showed hemolytic activity. In addition, the CCUG 998 and CNP 160 (AF012090) reference strains were classified as biotype Heyl [8], while CCUG 26451 and NCTC 10827 (AF224296) were classified as biotype Jawetz [15]. All 7 type E isolates were derived from rats and showed hemolytic activity on sheep blood agar. The most variable region of the 16S rDNA gene among the sequenced strains of *P. pneumotropica* is shown in Fig. 2. In this region, all the sequenced strains within each type were identical, except for the nucleotide sequence of AF224296 in type D. These results suggested that the genetic diversity of the *P. pneumotropica* isolates was complex; however, the *P. pneumotropica* isolates were phylogenetically discriminated based on host associations and β-hemolytic activity. Nakagawa *et al.* [14] reported that *P. pneumotropica* isolates from a rat could not be completely

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AF224296           1008:AGAGATGAGTTTGTGCCTTCGGGAACTTAGAGA:1040
Type A (PP26)       904:......ATAG.........A.......CT....: 936
Type B (PP9)        904:......ATTGGA.......................: 936
Type C (PP25)       904:......AGTC.....................T....: 936
Type D (CCUG26451)  904:......AC.GGA............G...T....: 936
Type E (PP19)       905:......ATTGGA.......A.......G......: 937
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Fig. 2. Polymorphic nucleotide sites of the most variable region among the 16S rDNA alleles of the sequenced strains of *P. pneumotropica*. The dots represent the nucleotides present in the topmost sequence (AF224296).

transmitted to a mouse, while isolates from a mouse could be completely transmitted to a rat. In addition, *Haemophilus* spp., which is closely related to *P. pneumotropica* and prefers V-factor in the media, could be transmitted from rats to mice and from guinea pigs to rats, but could not be transmitted from guinea pigs to mice [2]. Recently, *P. pneumotropica* isolates and their closely related species were divided into six clusters based on the 16S rDNA sequence [7], and the phylogenetic analysis in this study also discriminated similar clusters. Furthermore, housekeeping genes and 16S rDNA sequences have indicated that the members of *Pasteurellaceae* are classified on the basis of their host associations [4, 5, 10]. Therefore, *P. pneumotropica* infections are considered to be host specific. Although *P. pneumotropica* was classified as biotypes Jawetz and Heyl based on carbon source and amino acid utilization [8, 12, 15], phylogenetic analysis revealed that significant differences could not be confirmed among them. Their genetic relationships might be more heterogenous than their phenotypic characteristics. Although the diversity of *P. pneumotropica* isolates might increase in the future, phylogenetic analysis based on specific gene sequences might be more definite than biochemical properties. The isolates that showed hemolytic activity were divided into 2 types according their phenotypes. Namely, the isolates that belonged to type B showed hemolytic activity on horse blood agar, but not sheep blood agar, while the isolates that belonged to type E showed hemolytic activity on sheep blood agar, but not horse blood agar. Both of the hemolytic activities were stabilized on horse or sheep blood agar through several passages. Characterization and distribution of both hemolysins should be clarified. The infectious agents in rodents related to *P. pneumotropica* are *Actinobacillus muris* and *Haemophilus influenzaemurium*, which both belong to the *Haemophilus-Pasteurella-Actinobacillus* (HPA) complex. A comparison of these strains, which are deposited in GenBank (*Actinobacillus muris* NCTC 12432, AY362894; *Haemophilus influenzaemurium*, AF024530), with the *P. pneumotropica* isolates showed that both *A. muris* and *H. influenzaemurium* were closely related to the strains that belong to type D (data not shown). No specific differences in the 16S rDNA genes existed among the HPA complex. In the case of identification of *P. pneumotropica*, the HPA complex could not be strictly discriminated by only 16S rDNA gene analysis, and therefore, identification of *P. pneumotropica* should also require biochemical analysis. The phylogenetic differences among the HPA complex should be clarified in the future studies.

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