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Evaluation of PCR as a Means of Identification of *Pasteurella pneumotropica*

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Abstract: A polymerase chain reaction with new primers (new PCR) designed from *Pasteurella pneumotropica* 16S rDNA as an identification system for this organism was compared with the PCR reported by Wang *et al.* (Wang's PCR) by using 15 bacterial reference species and 70 clinical isolates with the conventional identification system. For the 15 reference strains, both PCRs were identical. For the 70 clinical isolates, the new PCR and Wang's PCR showed consistency with the conventional system in 62.9% (44/70) and 51.4% (36/70), respectively. Twenty-six isolates were inconsistent with the conventional system and the new PCR with respect to morphology and serology. These findings suggested that the new PCR was more sensitive than Wang's PCR, and the new PCR in combination with morphology and serology is useful for *P. pneumotropica* identification.

Key words: identification, PCR, *P. pneumotropica*

Although *Pasteurella (P.) pneumotropica* is associated with the respiratory tract and genital tract of mice, rats, hamsters and guinea pigs [1–5] and was classified as a primary pathogen of these laboratory animals, its significance as a pathogen is not considered to be high [1–4, 6]. When animals colonized with this organism were co-infected with murine pathogens such as *Mycoplasma pulmonis* or Sendai virus, the severity increased [2, 3]. This organism colonizes the nasopharynx, oral cavity, upper respiratory tract, conjunctiva, urinary bladder, skin, mammary glands, genital tract, and intestines [1, 3–8]. The infected animals have usually been detected by culture of the upper respiratory tract [5, 6] and/or swab specimens of the conjunctiva taken with

blood agar plates. *P. pneumotropica* has been identified from the morphology by Gram staining of typical colonies, biochemical characteristics and the slide agglutination test with antiserum [5]. The conventional identification system of this organism presents problems because of its subjective evaluation and the long time required. Recently 16S rRNA sequences have been used to determine phylogenetic relationships between eukaryotic and prokaryotic organisms [9], and to detect pathogens from various materials by specific amplification of ribosomal sequences [10, 11]. A polymerase chain reaction (PCR) method to detect *P. pneumotropica* was also reported by Wang *et al.* [1]. In this study, the new PCR primers which we designed

(Received 27 February 1998 / Accepted 11 August 1998)

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to identify *P. pneumotropica* were evaluated by comparing with the PCR previously reported by Wang *et al.* (Wang's PCR).

To evaluate the usefulness of PCR for the identification of *P. pneumotropica*, 85 strains were used in this study. They were 15 standard strains (*P. pneumotropica* ATCC 35149 and N8, *P. aerogenes* ATCC 29554, *P. gallinarum* ATCC 13361, *P. multocida* pm78, *Actinobacillus ureae* ATCC 29693, *Klebsiella pneumoniae* RE587, *Streptococcus (S.) pneumoniae* H473-1, *S. zooepidemicus* F921-4, *Escherichia coli* O115 yoken, *Bordetella bronchiseptica* yoken, *Mycoplasma (M.) arthritidis* PG6, *M. neurolyticum* Type A, *M. pulmonis* m53 and *Corynebacterium kutscheri* CK-1) and 70 clinical isolates originating in the upper respiratory tract and conjunctiva of laboratory animals and were maintained in our laboratory. These 70 clinical isolates were selected according to the following criteria: Gram-negative coccobacillus, forming grayish-white, smooth, nonhemolytic colonies about 3 mm in diameter on blood agar after incubation at 37°C for 48 hr. Thirty-four of the 70 isolates (28 from mice, five from rats, and one from a hamster) were identified as *P. pneumotropica*, and the remaining 36 isolates (10 from mice, 19 from rats, five from hamsters, two from guinea pigs) were identified as non-*P. pneumotropica* by the conventional system in our laboratory. This determined the morphological characteristics by Gram staining, the API-20 NE test (BioMérieux Vitek Japan Inc., Tokyo) and a slide agglutination test with rabbit sera hyperimmune to the *P. pneumotropica* N8 strain.

The sequence of primers for the new PCR and Wang's PCR are shown in Table 1. The design of the new PCR primers was based on the 16S rDNA sequences of *P. pneumotropica* (M75083) from the GenBank and that of the E435-38 strain isolated from a mouse in our laboratory. The new PCR product was a 296 base pair DNA fragment.

DNAs for PCR analysis were extracted by a modification of the method of Wang *et al.* [1]. Briefly, colonies formed on 5% horse blood agar at 37°C for 48 hr were suspended in PBS (–). After washing with PBS (–) and sterile distilled water, the organisms were suspended in 1% Triton X-100. The samples were heated at 100°C for 5 min, then centrifuged at 14,000 rpm for 10 min and stored at 4°C until use.

PCR analysis was performed as follows. Ten microliters of 10 fold diluted DNA was added to 40 μ l of a

PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphate, 1 μ M sense primer PPN-1 or PPF-1, 1 μ M antisense primer PPN-2 or PPR-2 and 5 units of *Taq* polymerase (Takara Shuzou Co., Kyoto) [9]. Each mixture was overlaid with mineral oil, and then placed in a DNA thermal cycler 480 (Perkin-Elmer Co., U.S.A.). PCR was performed by 25 cycles of denaturation at 93°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 1 min. The PCR amplification products were analyzed by gel electrophoresis in 4% agarose.

Two reference strains of *P. pneumotropica* (ATCC 35149 and N8) showed positive results and 13 strains of other bacterial species showed negative results in both the new PCR and Wang's PCR. Results of the new PCR and Wang's PCR with 70 isolates are shown in Table 2. The new PCR showed positive results for all 34 *P. pneumotropica* isolates identified by the conventional system and negative results in only 10 of 36 non-*P. pneumotropica* isolates. This meant that all 26 isolates identified as non-*P. pneumotropica* by the conventional system were classified as *P. pneumotropica* by the new PCR. On the other hand, Wang's PCR showed positive results for 18 out of 34 *P. pneumotropica* isolates and negative results for 18 of 36 non-*P. pneumotropica* isolates. Results of the new PCR and Wang's PCR were 62.9% (44/70) and 51.4% (36/70), respectively, which were consistent with those of the conventional system. As shown in Table 3, 26 isolates were inconsistent with the results obtained by the new PCR and the conventional system. Although all 26 isolates were positive in the new PCR, these isolates were different from *P. pneumotropica* either morphologically or serologically. The results of the biochemical evaluation were basically consistent with those of the new PCR.

The procedure that had been used to identify *P. pneumotropica* in our laboratory was morphological confirmation with Gram-staining in typical colonies of *P. pneumotropica* on horse blood agar, followed by the API-20 NE test and by a slide agglutination test using rabbit antiserum, but morphologic evaluation is subjective and identification by the API-20 NE test is time-consuming. In this study, out of 70 isolates, 34 *P. pneumotropica* isolates were consistent with the results obtained by the new PCR and the conventional test. Among 36 non-*P. pneumotropica* isolates, 26 isolates

Table 1. Sequences of primers used to amplify DNA segments of the 16S rRNA sequence of *P. pneumotropica*

Primer	Sequence (5'–3')	Position ^a
PPN-1 ^b	TTGCATTTCAGACTGGGAATC	624–644
PPN-2 ^b	GCACAAAACCTATCTCTAGTCTC	1018–997
PPF-1 ^c	ACCTACTCTTGACATCCAGA	974–993
PPR-2 ^c	CGCTCCACATCGCTGCTT	1269–1252

^a Positions within the *P. pneumotropica* 16S rRNA sequence (M75083) corresponding to the 5' and 3' ends of each primer.

^b Primers reported by Wang *et al.* 1996. ^c Primers used in this study.

Table 2. Comparison of results of *P. pneumotropica* with 70 isolates obtained from laboratory animals by the new PCR and Wang's PCR

	No. of isolates	new PCR		Wang's PCR	
		Pos.	Neg.	Pos.	Neg.
<i>P. pneumotropica</i> *	34	34	0	18	16
non- <i>P. pneumotropica</i>	36	26	10	18	18

* The organisms were identified by morphological, biochemical and serological evaluations.

Table 3. Results for isolates which were inconsistent with results obtained by the new PCR and the conventional identification system

Pattern No.	Morphology	Biochemistry ^a	Slide agglutination	No. of samples (Origin)
1	+ ^b	+	–	5 (rats)
2	± ^c	+	–	3 (rats)
3	– ^d	+	+	7 (6 mice, 1 rat)
4	– ^d	+	–	9 (1 mouse, 6 rats, 2 hamsters)
5	– ^d	–	+	1 (mouse)
6	– ^d	–	–	1 (rat)

^a The results of *P. pneumotropica* identification by the API-20NE test. ^b+: The colonies were about 3 mm diameter, grayish-white, smooth, nonhemolytic colonies on blood agar after incubation at 37°C for 48 hr, and the bacteria recognized as Gram-negative coccobacilli under a microscope. ^c±: Although the colonies were typical of *P. pneumotropica*, the length of the organisms was different from that of the reference strains. ^d–: Length of organisms was different from that of reference strains.

were identified as *P. pneumotropica* by the new PCR. Although most of them were also classified as *P. pneumotropica* by the API-20 NE test, they were not identified as *P. pneumotropica* from a combination of morphology and the slide agglutination test. These findings suggested that the new PCR may replace biochemical evaluation for identification of *P. pneumotropica*.

Sixteen (47.1%) out of 34 *P. pneumotropica* isolates could not be identified as *P. pneumotropica* by Wang's PCR, although Wang's PCR showed higher specificity than the new PCR (Table 2). The SDS-PAGE band pattern of these isolates identified as *P. pneumotropica* by the conventional method and as non-*P. pneumotropica* by Wang's PCR was identical to that of standard *P. pneumotropica* (ATCC 35149) (data not shown). We found that 16S rDNA sequences of a database (M75083) and E435-38 were different in the region of the antisense primer (PPN-2) as shown in Fig. 1. This appeared to be the reason why Wang's PCR did not amplify the DNA of this strain. The new PCR amplified *P. pneumotropica*

M75083 E435-38	620	630 (PPN-1)	640	650	660
	CTTAACCTGG	GAATTCATTCAGACTGGG	AATCTAGAGT	ACTTTAGGGA	
M75083 E435-38	970	980 (PPF-1)	990	1000 (PPN-2)	1010
	CGCGAAGAAC	CTTACCTACTCTTGACATCC	AGAGAAGAGA	CTAGAGATAG	
M75083 E435-38	1020	1030	1040	1050	1060
	TTTCTGCT	TAGGGAACCTT	GAGACAGT	GCTGCATGGC	TGTCGTCAGC
M75083 E435-38	1250 (PPR-2)	1260	1270	1280	1290
	AGAGGGAAGC	GAGGAGCGA	TGTGGAGCA	ATCTCATAAA	GTGCGTCTAA

Fig. 1. Comparison of DNA sequences in *P. pneumotropica* M75083 and the E435–38 segment corresponding to PCR primers and their neighbors. Identical (*) nucleotides are indicated.

DNA in all of the isolates.

In conclusion, a PCR to identify *P. pneumotropica* was developed. This PCR has a higher sensitivity than Wang's PCR and is useful for the identification of *P. pneumotropica* in combination with morphology and the slide agglutination test.

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