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Construction of a DNA Probe and Detection of *Actinobacillus pleuropneumoniae* by Using Polymerase Chain Reaction

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A 1.5-kb *Actinobacillus pleuropneumoniae* 4074 DNA fragment from a genomic library was found to hybridize to DNA from the 12 *A. pleuropneumoniae* serotypes by Southern hybridization. No cross-hybridization was detected with DNAs from hemolytic members of the family *Pasteurellaceae*. From the nucleotide sequence of the putative genomic probe, three primers were synthesized for use in polymerase chain reactions (PCRs), with 31 strains tested by using purified and crude DNA targets. PCR amplification products of 610 and 985 bp were observed in nucleic acids extracted from the 12 known serotypes and a biotype 2 strain. Template DNAs from other gram-negative and gram-positive bacteria, some of them found in the normal flora of swine and the upper respiratory tract, were not amplified by PCR. The only exception was an amplification of a similar 610- or 985-bp sequence in *Actinobacillus lignieresii*, a species that is closely related to *A. pleuropneumoniae* but that has never been isolated from swine. Amplification of specific *A. pleuropneumoniae* sequences by PCR directly from clinical specimens may find applications in the identification of asymptomatic carriers as well as in efforts to eradicate porcine pleuropneumonia.

Porcine pleuropneumonia, a major contagious respiratory disease in pigs, is distributed worldwide and causes severe economic losses to the swine industry (30). The disease is characterized by fibrinous pleuritis, with hemorrhagic and necrotic lesions in the lungs (19); clinical symptoms and pathologic lesions found at the time of necropsy lead to a clear diagnosis. The causal agent of porcine pleuropneumonia, *Actinobacillus pleuropneumoniae*, is a gram-negative pleomorphic coccobacillus that is considered to be a porcine-specific pathogen. At least 12 serotypes with various degrees of virulence have been identified (5). The disease appears to be increasing in prevalence because of intensified production practices. Despite the growth requirement for NAD (or V factor), *A. pleuropneumoniae* is easy to grow (31).

Animals that survive an *A. pleuropneumoniae* infection generally suffer from chronic lesions and become subclinical carriers of the pathogen. The ideal protocol for detection of *A. pleuropneumoniae* would be to culture organisms from healthy animals; however, this traditional approach has not been successful (8, 12, 13, 17) and is time-consuming when one is dealing with several herds and hundreds of animals. Detection of the bacterium from live animals by a sensitive method would be useful for identifying *A. pleuropneumoniae* cells within the normal flora of the upper respiratory tract directly from a nasal or tonsil swab.

Methods have been described which permit the enzymatic amplification of specific DNA sequences (28) that can be used to increase a single copy of a given DNA target (template) sequence by 10^{12}-fold (14). Over the past few years, the polymerase chain reaction (PCR) has become the method of choice for amplifying DNA and RNA for a variety of purposes. The PCR procedure, which uses specific oligonucleotide primers and *Taq* DNA polymerase, can produce a specific DNA fragment from mixed microbial specimens (7). The PCR system can detect a single microorganism and can be used on mixed microbial specimens without the isolation of individual species of bacteria (24).

The aim of this work was to develop such a sensitive and specific method, using PCR, to detect the presence of *A. pleuropneumoniae* DNA under conditions that would mimic those used for the analysis of clinical specimens.

MATERIALS AND METHODS

Bacterial strains and plasmids. A complete list of the bacterial strains and their sources used for probe testing and PCR analysis is given in Table 1. *Escherichia coli* JM101 [SupE *thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}ZΔM15)] was conserved on minimal medium plates without proline and was used for the construction of recombinants with the phagemid vectors pBGS18$^+$ and pBGS19$^+$ (Km^r *lacPOZ′*) (37). *E. coli* HB101 [F$^-$ *hsdS20* (r_b m_b) *SupE44 ara-14 galK lacY1 proA2 leu endA thi-2 rpsL20* (Sm^r) *xyl-5 mtl-1 recA13*] (2) was used for the preparation of chromosomal DNA and *E. coli* D1210 (derivative of HB101 *lacI*^{q}) was the recipient for the plasmid vector pOP203$A_2$$^+$ (Tet^r, A_2 gene from bacteriophage Qβ) (36). Bacterial cells were grown on tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing appropriate antibiotics (kanamycin, 50 μg/ml; tetracycline, 15 μg/ml).

Nucleotide sequencing. Single-stranded DNA was recovered from recombinant phagemids pPX1.5 and pXP1.5 by using the helper phage M13K07 (Pharmacia LKB Biotechnology, Baie d'Urfé, Québec, Canada) as described previously (33, 35). DNA sequencing was performed by the dideoxynucleotide T7 polymerase chain termination method (29) by using the universal primer and the T7 sequencing kit (Pharmacia). Analysis of the DNA sequence was performed with the software package of the University of Wisconsin Genetics Computer Group (4). Searches for similarities of the *A. pleuropneumoniae* 4074 genomic sequence with other DNA sequences were performed with the GenBank, European Molecular Biology Laboratory, and National Biomedical Research Foundation data bases.

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TABLE 1. Bacterial strains used for PCR analysis

Strain	Reference or source
Actinobacillus pleuropneumoniae 4074 (serotype 1)	26
Actinobacillus pleuropneumoniae 4226 (serotype 2)	18
Actinobacillus pleuropneumoniae 1421 (serotype 3)	18
Actinobacillus pleuropneumoniae M62 (serotype 4)	6
Actinobacillus pleuropneumoniae K17 (serotype 5a)	22
Actinobacillus pleuropneumoniae Femϕ (serotype 6)	27
Actinobacillus pleuropneumoniae WF83 (serotype 7)	27
Actinobacillus pleuropneumoniae 405 (serotype 8)	23
Actinobacillus pleuropneumoniae 13261 (serotype 9)	10, 20
Actinobacillus pleuropneumoniae 13039 (serotype 10)	21
Actinobacillus pleuropneumoniae 16153 (serotype 11)	R. Higgins
Actinobacillus pleuropneumoniae 1092 (serotype 12)	R. Higgins
Actinobacillus pleuropneumoniae 204/78 (biotype 2)	J. Nicolet
Actinobacillus equuli ATCC 19392[a]	
Actinobacillus lignieresii ATCC 19393	
Actinobacillus rossii 88-846A	R. Higgins
Actinobacillus suis ATCC 15557	
Actinomyces pyogenes ATCC 19411	
Bordetella bronchiseptica ATCC 19395	
Erysipelothrix rhusiopathiae ATCC 19414	
Escherichia coli HB101	2
Haemophilus influenzae T2494	16
Haemophilus parasuis ATCC 19417	
Haemophilus sp. taxon C CAPM 5111	J. Nicolet
Haemophilus sp. taxon "minor group" 202	J. Nicolet
Pasteurella haemolytica ATCC 33396	
Pasteurella multocida ATCC 12048	
Pseudomonas aeruginosa ATCC 27853	
Streptococcus agalactiae ATCC 13813	
Streptococcus bovis ATCC 9809	
Streptococcus suis NCTC 10234[b]	

[a] ATCC, American Type Culture Collection.
[b] NCTC, National Collection of Type Cultures.

Preparation of DNA templates for PCR. All *Haemophilus* and *A. pleuropneumoniae* strains were grown on chocolate agar containing 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). All other strains were grown on blood agar plates (tryptic soy agar with 5% sheep erythrocytes). DNA templates were prepared from an overnight culture on appropriate plates. A loopful of bacteria was resuspended in 100 μl of sterile distilled water, and the extraction of DNA was done by using a freezing-boiling technique (34). Purified chromosomal DNA was prepared as described previously (32).

Amplification protocol. Reagents used for PCR amplification (GeneAmp kit) were purchased from Perkin-Elmer Cetus (Norwalk, Conn.), and mixtures were prepared according to the instructions of the manufacturer. Samples of 100-μl reaction mixtures were amplified in the DNA thermal cycler (Perkin-Elmer Cetus), which was programmed for 25 cycles of amplification. Parameters for the amplification cycles consisted of 1 min at 94°C (denaturation), 2 min at 37 or 50°C (primer annealing), and 3 min at 72°C (primer extension-polymerization). An aliquot of the reaction mixture (10 μl) was analyzed by agarose gel (1 to 1.5%) electrophoresis, and the products were stained with ethidium bromide and visualized under UV light.

RESULTS

Construction of the DNA probe. A genomic library of strain *A. pleuropneumoniae* 4074 was constructed by partially digesting chromosomal DNA with *Sau*3A and ligating it with *Bgl*II-digested pOP203A_2^+. The ligation mixture was transformed into *E. coli* D1210, and recombinants were selected on blood agar plates containing tetracycline and 0.5 mM isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co., St. Louis, Mo.) to eliminate nonrecombinant plasmids. From a collection of 4.5×10^3 colonies, a hemolytic recombinant clone was chosen for further characterization. The recombinant plasmid that was selected, pMON4180, was physically mapped with restriction endonucleases and contained a 2.7-kb insert. A 1.5-kb *Xho*I-*Pvu*II restriction fragment was isolated from pMON4180 and tested as a probe. The Southern hybridization experiments showed a strong hybridization signal at high stringency (32) with *Xho*I-*Pvu*II-restricted genomic DNAs from the 12 serotypes of *A. pleuropneumoniae*. The probe tested negative against genomic DNA preparations from the following hemolytic members of the family *Pasteurellaceae*: *A. equuli*, *Actinobacillus rossii*, *A. suis*, and *P. haemolytica* (data not shown).

Nucleotide sequence of the *Xho*I-*Pvu*II restriction fragment. The 1.5-kb DNA probe fragment from pMON4180 was subcloned into the *Sal*I-*Sma*I site of the pBGS18$^+$ plasmid vector polylinker. For subcloning into the opposite orientation to determine the sequence of the complementary DNA strand, the plasmids pBGS18$^+$ (1.5-kb *Xho*I-*Pvu*II insert) and pBGS19$^+$ were digested with *Eco*RI-*Hin*dIII and ligated together. Selection for kanamycin-resistant recombinants was based upon selection of clear versus blue plaques in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Bethesda Research Laboratories Inc., Gaithersburg, Md.). The nucleotide sequences of subcloned fragments were determined by using a single-stranded DNA template isolated from both pBGS18$^+$ and pBGS19$^+$ recombinants with the universal primer. More than 350 bases of sequence were determined by using each end of the *Xho*I-*Pvu*II genomic probe. The nucleotide sequence that was obtained showed no significant homology with other DNA sequences available in data banks.

Selection of primers. From the last 50 nucleotides read, two 17-mer oligonucleotide primers, PX-1 and XP-1, were synthesized to determine the complete sequence of the *Xho*I-*Pvu*II fragment. One 19-mer oligonucleotide primer, APL-1, was synthesized (Table 2) for PCRs with genomic DNAs from the strains listed in Table 1. In addition, two 20-mer oligomers, which were used as broad-range eubacterial primers (Table 1) (3) homologous to conserved eubacterial 16S RNAs, were synthesized. These last two primers were used to test the capability of obtaining PCR products directly from crude templates.

Amplification of samples. From the freezing and boiling of crude samples, 5-μl aliquots were mixed with the prokaryotic broad-range primers, to test for amplification of a 226-bp DNA fragment within the 16S rDNA. The 14 bacterial species used tested positive; the results that were obtained are shown in Fig. 1. The annealing temperature of 37°C was used because of the possible divergence of nucleotide sequences among diverse bacterial species. Similar results were obtained when purified and crude DNA templates from all strains listed in Table 1 were used. Figure 2 shows the positive amplifications visualized as the expected 985-bp fragments for the 12 known *A. pleuropneumoniae* serotypes by using the XP-1 and APL-1 PCR primers. Results of preliminary experiments performed to confirm the specificity of *A. pleuropneumoniae* XP-1 and APL-1 primers and the 985-bp products with purified DNA templates are shown in

TABLE 2. Oligonucleotide primers used in this study

Primer name[a]	Sequence	Reference
PX-1	5′-CCGACTTTTAAATCCGT-3′ (17-mer)	This study
XP-1	5′-GAACAGTTGTTCGCTAA-3′ (17-mer)	This study
APL-1	5′-CCGCTTCCGTACAGCAATC-3′ (19-mer)	This study
p11p complement	5′-GAGGAAGG$^{A}_{T}$GGGGA$^{C}_{T}$GACGT-3′ (20-mer)	3
p13p	5′-AG$^{G}_{A}$CCCG$^{G}_{A}$GAACG$^{T}_{G}$ATTCAC-3′ (20-mer)	3

[a] The primers PX-1, XP-1, and APL-1 were selected from the 1.5-kb *Pvu*II-*Xho*I DNA fragment of *A. pleuropneumoniae* 4074. The p11p complement and p13p primers were derived from conserved eubacterial 16S rDNA.

Fig. 3. Nucleic acid amplification was positive only with *A. pleuropneumoniae* template DNA (Fig. 3, lane B).

With the same amount of crude template as was used for rDNA broad-range primers, PCR was done with templates obtained from 14 strains by using XP-1 and APL-1 primers, and results are shown in Fig. 4. We noted three bands for *A. pleuropneumoniae* 4074, *A. lignieresii*, and *A. pleuropneumoniae* biotype 2 (Fig. 4, lanes B, E, and O, respectively). Additional PCRs confirmed a 610-bp product with the XP-1 and PX-1 primers for the 12 serotypes, biotype 2, and *A. lignieresii* (data not shown).

Purified DNA template as well as crude material could be amplified by the PCR method, as demonstrated by the eubacterial universal primers and the positive reactions with specific oligomers. However, we noted variations in the concentrations of amplified product for a given set of primers (for example, in Fig. 1, lanes F, G, and N, and Fig. 2, lanes A, B, E, and F). This was presumably caused by differences in the quality and concentration of template DNA obtained from distinct species of bacteria, which can affect optimization of PCR conditions.

DISCUSSION

The definitive diagnosis of porcine pleuropneumonia is based on clinical symptoms, typical lung lesions, and isolation of the causative agent, *A. pleuropneumoniae* (15). Identification of a previous or chronic infection requires serological testing (15). Cultivation of *A. pleuropneumoniae* from lungs obtained at the time of necropsy is the standard method of detection, although it has not always been possible to detect the agent from other clinical specimens (12, 13, 17, 25). The normal flora of the upper respiratory tract is thought to interfere with the process of convenient isolation of *A. pleuropneumoniae* from live animals, especially if few cells of *A. pleuropneumoniae* are present in the specimen. The DNA detection system described in this report recognized and detected all 12 serotypes tested, in addition to a biotype 2 (V factor-independent) strain; thus, the assay showed a discriminating capacity for all types of strains studied which can be designated *A. pleuropneumoniae*. Interestingly, no amplification was obtained with the *Haemophilus* sp. taxon "minor group" strain 202, which represents a member of a distinct group (11). The same oligomer combinations, XP-1–PX-1 and XP-1–APL-1, could also recognize and amplify DNA from a closely related bacterial species, *A. lignieresii*. In fact, *A. lignieresii*, the type species of the genus *Actinobacillus*, has been shown to be closely related by DNA homology to *A. pleuropneumoniae* (26). This sole cross-amplification was also obtained at an annealing temperature of 50°C, but it was not limiting because the latter bacterium is not encountered in swine.

In our case, the broad-range oligomers p11p complement and p13p, which were directed toward the 16S eubacterial

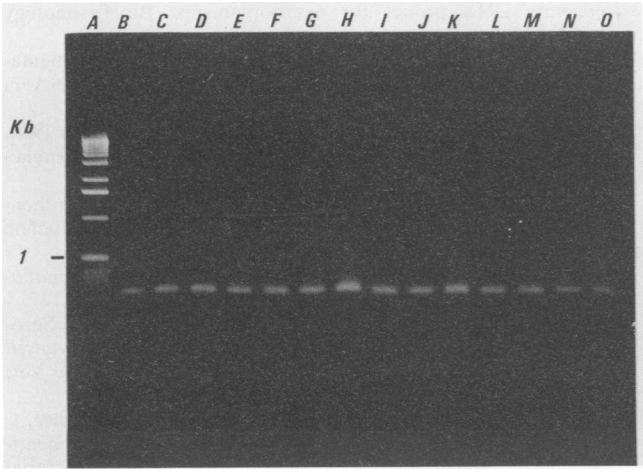

FIG. 1. Agarose gel (1.5%) electrophoresis of nucleic acid amplification products from crude DNA template with rDNA broad-range primers. Lanes: A, 1-kb DNA ladder (Bethesda Research Laboratories); B, *A. pleuropneumoniae* 4074; C, *P. multocida*; D, *E. rhusiopathiae*; E, *A. lignieresii*; F, *S. suis*; G, *S. agalactiae*; H, *P. aeruginosa*; I, *A. pyogenes*; J, *S. bovis*; K, *B. bronchiseptica*; L, *Haemophilus* sp. taxon C; M, *Haemophilus* sp. taxon "minor group"; N, *H. parasuis*; O, *A. pleuropneumoniae* biotype 2.

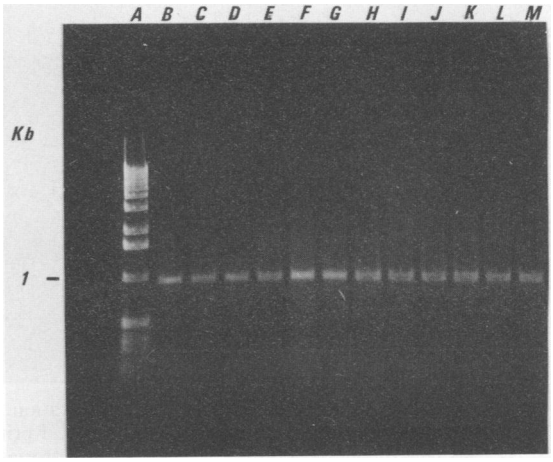

FIG. 2. Agarose gel (1%) electrophoresis of nucleic acid amplification products from crude DNA templates with the XP-1 and APL-1 primers. Lanes: A, 1-kb DNA ladder (Bethesda Research Laboratories); B to M, *A. pleuropneumoniae* serotypes 1 to 12, respectively.

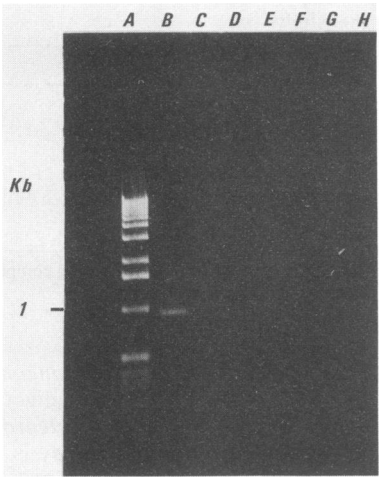

FIG. 3. Agarose gel (1%) electrophoresis of nucleic acid amplification products purified DNA templates with XP-1 and APL-1 primers. Lanes: A, 1-kb DNA ladder (Bethesda Research Laboratories); B, *A. pleuropneumoniae* 4074; C, *H. influenzae*; D, *A. equuli*; E, *E. coli*; F, *A. suis*; G, *Actinobacillus rossii*; H, *P. haemolytica*.

ribosomal subunit, were found to be useful and confirmed the DNA amplification products obtained from crude templates. Amplification of the expected ca. 226-bp DNA fragment was a prerequisite to carry on any subsequent amplification tests with the specific primers, PX-1, XP-1, and APL-1. Eubacterial broad-range oligomers showed that a wide variety of bacterial species can be detected in a PCR assay. In fact, we were able to detect 16 additional species not described in the previous work (3). Moreover, we confirmed that numerous cells ($>10^6$/ml) could inhibit the PCR, as already described elsewhere (1). These experiments were useful as controls to avoid the false-negative results

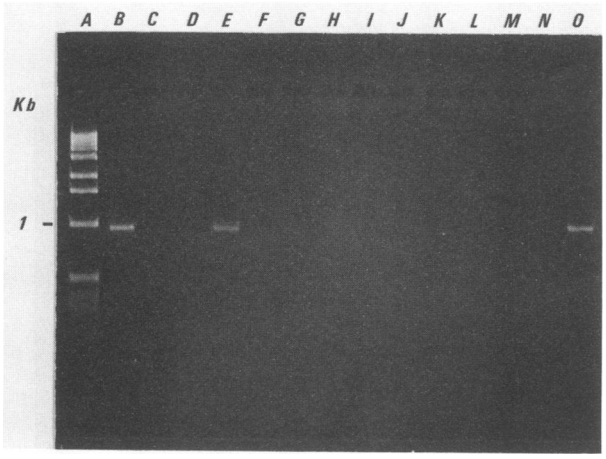

FIG. 4. Agarose gel (1%) electrophoresis of amplification products from crude DNA templates with the XP-1 and APL-1 primers. Lanes: A, 1-kb DNA ladder (Bethesda Research Laboratories); B, *A. pleuropneumoniae* 4074; C, *P. multocida*; D, *E. rhusiopathiae*; E, *A. lignieresii*; F, *S. suis*; G, *S. agalactiae*; H, *P. aeruginosa*; I, *A. pyogenes*; J, *S. bovis*; K, *B. bronchiseptica*; L, H, taxon C; M, *Haemophilus* sp. taxon "minor group"; N, *H. parasuis*; O, *A. pleuropneumoniae* biotype 2.

encountered in PCR amplifications of crude DNA templates caused by inhibitors (9).

We identified three primers as potential candidates for use in an *A. pleuropneumoniae*-specific detection system on the basis of nucleic acid amplification. Further testing with genomic DNA isolated from other *Actinobacillus* species and closely related genera like *Haemophilus* and *Pasteurella* confirmed the almost absolute specificity of the amplification. Since the specificity of the reaction has been confirmed, the sensitivity of the detection system must be assayed, starting with an *A. pleuropneumoniae* culture of known cell concentration in which dilutions could be prepared, bacterial cells lysed, and target DNA amplified. Such a technique has been used to detect small numbers of *Legionella pneumophila* bacterial cells in water samples (34). If it is assumed that this technique has adequate sensitivity, studies involving porcine lung biopsy specimens and nasal secretions need to be tested in amplification reactions.

We described the first steps in developing a sensitive and specific detection system for *A. pleuropneumoniae*. Further testing should give more knowledge and a better understanding of the prevalence and distribution of *A. pleuropneumoniae*. Prevention of the normal course of an epidemic would be beneficial for minimizing losses in the porcine industry.

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