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Serotype Identification of *Actinobacillus pleuropneumoniae* by Arbitrarily Primed Polymerase Chain Reaction†

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Rapid and accurate determination of the *Actinobacillus pleuropneumoniae* serotype involved in a disease outbreak is important both in limiting the severity of an outbreak and for tracing the source of the infecting organism. This study describes the use of arbitrarily primed polymerase chain reaction (AP-PCR) as a rapid, precise, and genetically based procedure to identify *A. pleuropneumoniae*. AP-PCR amplification of bacterial genomic DNA results in specific DNA profiles, which can be used to differentiate currently recognized serotypes. This technique is especially useful for identifying previously nontypeable and serologically cross-reactive *A. pleuropneumoniae* field isolates. Consecutive passages of isolates on different media, freezing, and subsequent infection of pigs did not alter the AP-PCR genomic profile. We propose the use of M13 and T3-T7 oligodeoxynucleotide primers for diagnostic and epidemiological identification of *A. pleuropneumoniae* by AP-PCR techniques.

Actinobacillus pleuropneumoniae is the etiologic agent of contagious porcine pleuropneumonia, a severe and often fatal respiratory disease of pigs (13, 23, 29). Pigs that survive infection with one serotype of *A. pleuropneumoniae* develop resistance to infection by any other serotype (15, 16), although surviving animals often become asymptomatic carriers. Immunization reduces mortality and severity of the disease but routinely fails to prevent infection (15, 18). Immunized animals may become asymptomatic carriers of the organism (15), and, because of the serotype specificity of vaccination-induced immunity (15–18), these pigs can be infected by other *A. pleuropneumoniae* serotypes from other asymptomatic carriers.

Twelve serotypes of *A. pleuropneumoniae* biotype 1 (V-factor dependent) have been identified on the basis of immunologically unique capsular and lipopolysaccharide antigens (1, 3, 6, 12, 14, 19–24, 26). Additionally, two serotypes of biotype 2 (V-factor independent) have also been identified (2). Several serotypes cross-react serologically, i.e., serotypes 1, 9, and 12; serotypes 4 and 7; and serotypes 3, 6, and 8 (14). These cross-reactive serotypes can be typed definitively only by indirect hemagglutination (4, 8). Self-agglutinating and nontypeable field strains of *A. pleuropneumoniae* are also common (5, 9, 10).

Serotype identification is important in studying the epidemiology of *A. pleuropneumoniae* because the prevalence of specific serotypes varies with geographic location (11, 14, 25). In the United States, for example, serotypes 1, 5, and 7 predominate (25). In Canada, serotypes 1, 3, and 5 are frequent isolates (11, 28), whereas in Scandinavia and Europe, serotypes 1, 2, and 9 are most frequently isolated (2, 7). Serotyping *A. pleuropneumoniae* provides a means of identifying when new serotypes enter a particular locale, thus informing producers what necessary steps must be taken to protect pigs not previously exposed to the organism or vaccinated against the new serotype.

The polymerase chain reaction is a powerful tool for amplifying specific DNA fragments for a variety of purposes. The use of arbitrary primers at initial low-stringency amplification (31, 32) for fingerprinting of highly complex genomes provides a means to evaluate genetic relatedness between organisms of different genera as well as different species. The arbitrarily primed polymerase chain reaction (AP-PCR) technique has been theoretically compared with enzyme isotyping as a tool to evaluate relatedness between bacterial strains (31, 32).

We examined AP-PCR as a rapid and reliable method of genetically defining the serotype identity of *A. pleuropneumoniae*. We propose the use of specific oligodeoxynucleotide primers and uniquely sized DNA fragments as a routine PCR technique for determining the identity of *A. pleuropneumoniae* isolates.

MATERIALS AND METHODS

Primers and primer synthesis. The Universal M13 primer (TTATGTAAAACGACGGCCAGT), M13 reverse-sequencing primer (GGAAACAGCTATGACCATG), T7 primer (GTAATACGACGCACTATAG), and T3 primer (GCAATTAACCCTCACTAAAG) were used in this study. Standard phosphoramidite chemistry was used to synthesize all oligodeoxynucleotide primers on a model 391 PCR-Mate DNA synthesizer (Applied Biosystems). Primers were removed from columns with 100% ammonium hydroxide, and protective groups were removed by heating at 55°C for 5 h. Primers were dried under vacuum and resuspended in 500 μl of double-distilled water. Gel filtration on Sephadex G-50 columns (Nick columns; Pharmacia LKB) was used for purification of oligodeoxynucleotides. Primers were quantified on a UV-Vis spectrophotometer (Gilford RESPONSE) at 260 nm.

DNA preparation. Reference strains of *A. pleuropneumoniae* serotypes 1 to 12 were grown overnight at 37°C on enriched chocolate agar plates (Micro Diagnostics). For DNA preparation, single colonies were boiled in 25 μl of water for 5 min before being pelleted in a Microspin 24S

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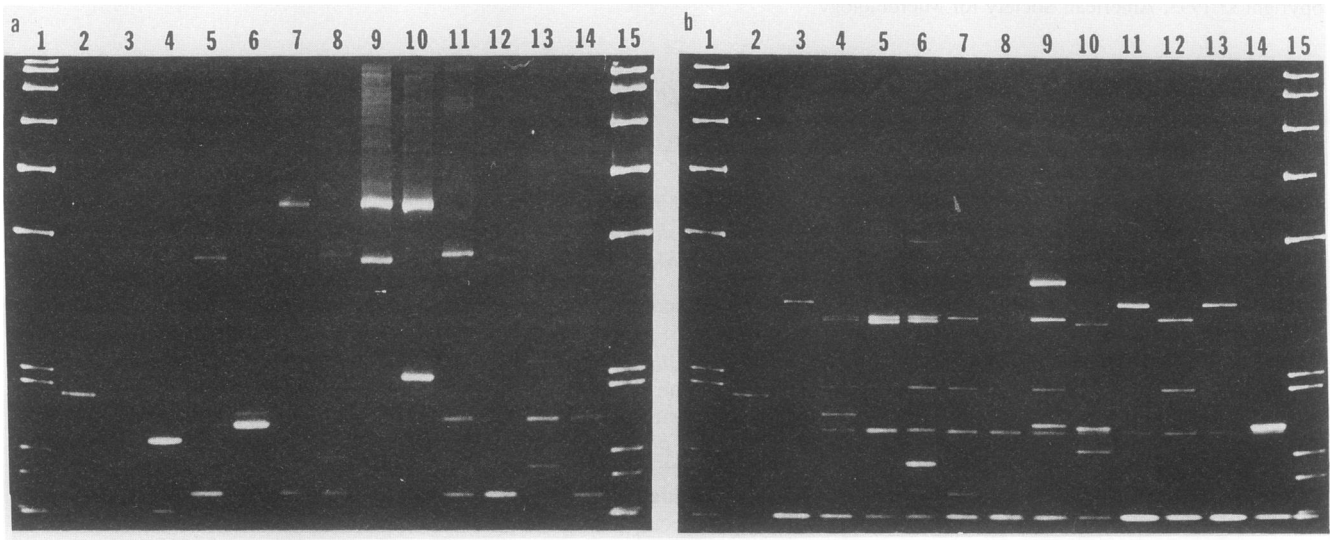

FIG. 1. Genomic fingerprints of 12 reference serotypes of *A. pleuropneumoniae* amplified by AP-PCR and the M13 (a) and T3-T7 (b) primer pairs. A single colony from each serotype (grown on enriched chocolate agar) was boiled in 25 μl of double-distilled H_2O for 5 min; 5 μl of supernatant was used as DNA template for each amplification reaction. Lanes: 1, DNA ladder; 2, Perkin-Elmer Cetus DNA control; 3 to 14, *A. pleuropneumoniae* reference serotypes 1 through 12, respectively. Amplified products were resolved by electrophoresis on a 6% nondenaturing polyacrylamide gel and stained with ethidium bromide. Size markers are the 1-kb ladder from Bethesda Research Laboratories-GIBCO.

centrifuge (Sorvall). Supernatants (5 μl) from these samples were used as the DNA source for PCR amplification.

Bacterial strains and growth conditions. Reference strains of *A. pleuropneumoniae* representing serotypes 1 through 12 were S4074, S4226, S1421, M62, K17, Femo, WF83, 405, CVJ13261, D13039, 56153, and 8329, respectively. All reference serotypes were gifts of K. R. Mittal, University of Montreal, Saint-Hyacinthe, Quebec, Canada. *Actinobacillus suis*, hemolytic *Escherichia coli*, *Haemophilus parasuis*, and toxigenic *Pasteurella multocida* were isolated from clinical infections in pigs at the Kansas State University Diagnostic Laboratory. All bacteria were stored frozen at −70°C and grown overnight at 37°C on IsoVitaleX enriched chocolate agar plates (Remmel, Lenexa, Kans.).

Plasmid identification. *A. pleuropneumoniae* serotypes 1, 5, and 7 were evaluated for plasmids by CsCl-ethidium bromide density gradient centrifugation (27). Other *A. pleuropneumoniae* isolates were examined for plasmids by using a commercial plasmid minipreparation system (Magic Minipreps; Promega).

PCR. Reactions were performed in 100-μl volumes containing 4 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 0.001% gelatin; 1 mM (each) dATP, dCTP, dGTP, and TTP (Perkin-Elmer Cetus); 1 μM primers; 5 μl of DNA (described above); and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus). Amplification was performed in a Coy model 60 tempcycler programmed for 2 cycles of 5 min at 94°C, 5 min at 40°C, and 5 min at 72°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, using the fastest available ramp times between temperatures. Reaction amplification products were analyzed by electrophoresis on nondenaturing 6% polyacrylamide gels with a 50-μl sample volume and visualized by ethidium bromide staining of DNA.

Laboratory and animal passage of *A. pleuropneumoniae*. *A. pleuropneumoniae* reference serotype 1 was consecutively passaged in the laboratory 10 times on enriched chocolate agar plates and five times on blood-agar plates cross-streaked with *Staphylococcus aureus*. DNA prepared from individual colonies from each passage was amplified by using the M13 and T3-T7 primer sets as described (31).

Three crossbred pigs that were approximately 10 weeks old were infected intranasally with *A. pleuropneumoniae* serotype 1 (10^8 cells). Upon development of clinical signs of infection, pigs were humanely killed, and lung tissue was collected for bacterial culture. DNA from individual colonies of cultured *A. pleuropneumoniae* from lung tissue was amplified and compared with DNA from the preinfection culture.

RESULTS

AP-PCR DNA-generated profiles of *A. pleuropneumoniae* distinguish currently recognized serotypes. DNA from the 12 reference serotypes of *A. pleuropneumoniae* was amplified by AP-PCR. Distinct and specifically sized DNA fragments provided unique patterns that specifically distinguished each serotype, by using either a single primer or pairs of primers. AP-PCR-amplified DNA profiles from *A. pleuropneumoniae* reference serotypes 1 through 12, by using pairs of M13 and T7-T3 primers, are shown in Fig. 1a and b, respectively. Amplification of each serotype yielded at least one discreet DNA band that was unique for its identification. Plasmid DNA was not identified in the *A. pleuropneumoniae* reference serotypes or in the field strains used in this study; therefore, the profiles are specific for *A. pleuropneumoniae* genomic DNA.

A single primer was also used to differentiate serotypes of *A. pleuropneumoniae*. Reference strains of *A. pleuropneumoniae* amplified with the 5′-to-3′ Universal M13 primer sequence displayed unique amplification products, which were able to differentiate serotypes 1 and 9; serotypes 4 and 7; and serotypes 3, 6, and 8 (Fig. 2). These groups are serologically related and often cross-react with classic sero-

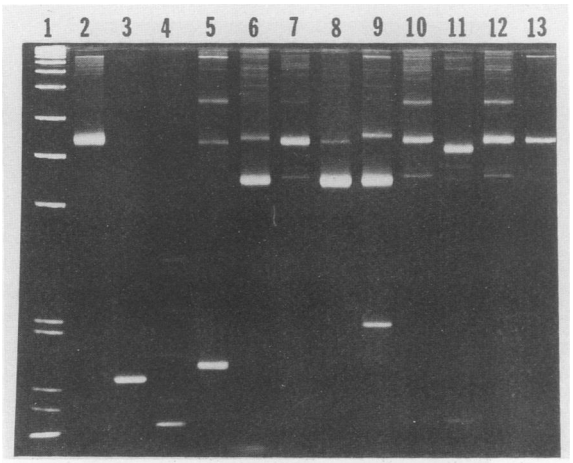

FIG. 2. Genomic fingerprints of 12 reference serotypes of *A. pleuropneumoniae* by using a single 5'-to-3' Universal M13 primer and AP-PCR. Lanes: 1, DNA ladder; 2 to 13, *A. pleuropneumoniae* serotypes 1 to 12, respectively. Amplified products were resolved by electrophoresis on a 6% nondenaturing polyacrylamide gel and stained with ethidium bromide. The size marker is the 1-kb DNA ladder from Bethesda Research Laboratories-GIBCO.

typing techniques. Table 1 lists AP-PCR DNA fragment sizes for each *A. pleuropneumoniae* reference serotype when amplified by AP-PCR on the Coy model 60 tempcycler, by using the conditions described with the M13 5' primer or the M13 primer.

AP-PCR DNA-generated profiles of *A. pleuropneumoniae* strains are unique from patterns similarly generated by other disease-causing gram-negative bacteria of pigs. Other important gram-negative bacterial pathogens of swine were examined by DNA AP-PCR amplification and compared with *A. pleuropneumoniae* reference serotype profiles. DNA from *A. suis*, *E. coli*, *H. parasuis*, and *P. multocida* was amplified by using the M13 (Fig. 3) and the T3-T7 primer pairs (data not shown) as described above and compared with all 12 serotypes of *A. pleuropneumoniae*. In all cases, DNA profiles distinct from *A. pleuropneumoniae* serotype profiles were elicited from the other bacteria.

Passage of bacteria in vitro and in vivo does not alter the AP-PCR-derived genomic profile. Consecutive passage of *A. pleuropneumoniae* on enriched chocolate agar and/or blood-agar plates did not alter the AP-PCR profiles by using the M13 and T7-T3 primer pairs (data not shown). Additionally, infection of pigs with a laboratory reference strain of *A. pleuropneumoniae* serotype 1, passaged multiple times in the laboratory, did not result in alteration of the AP-PCR generated DNA profile (data not shown).

Seven serotype 1 and 12 serotype 5 field isolates, which had been collected during the past 5 years at Kansas State University, were randomly selected for evaluation by AP-PCR. In all cases, field isolates that had been typed serotype 1 or serotype 5 by slide agglutination assay produced DNA profiles that, compared with the serotype specific AP-PCR reference patterns, distinguished their particular serotype in a blind assay. Additionally, strains that were nontypeable by conventional serotyping were easily compared with each other for epidemiological purposes after evaluation of their AP-PCR DNA profiles (Fig. 4).

TABLE 1. Molecular size map of fragments resulting from AP-PCR amplification of *A. pleuropneumoniae* reference serotypes 1 through 12[a]

Primer and serotype	Amplification fragment size (bp)
M13 (5')	
1	350, 525, 700, **1,800**
2	**400**
3	**310**, 350, 450, 525, 700
4	**425**, 1,400, 1,636, 1,800, 2,600
5	**1,300**, 1,850
6	1,350, 1,400, **1,800**, 2,600
7	**1,300**, 1,800
8	**517**, **1,300**, **1,850**
9	1,350, 1,400, **1,800**, 2,600
10	1,340, 1,410, **1,675**
11	1,350, 1,400, **1,800**, 2,600
12	1,340, 1,410, **1,800**, 2,600
M13 pair	
1	315, 350, 460, 1,000, 1,400, 1,450
2	**400**, 1,000
3	**300**, 1,000
4	**450**, 460
5	315, 350, 460, **1,300**
6	315, 350, 460, **1,300**
7	460, 520, **1,000**, **1,300**
8	**510**, **1,300**
9	315, 350, 460, 520, **1,000**
10	315, 350, 360, 1,000
11	350, 460, 520
12	315, 460, 1,000

[a] The primers used to obtain these profiles were either the M13 Universal forward primer (5') alone or the M13 primer pair, with the temperature-cycling protocol as described in Materials and Methods. Fragment sizes in boldface are the predominant DNA fragments amplified from each serotype by using the protocol and temperature cycler described in Materials and Methods.

DISCUSSION

Rapid and accurate determination of the *A. pleuropneumoniae* serotype involved in a disease outbreak is important both for limiting the severity of the outbreak and for tracing the source of the infecting organism. This study demonstrated the use of AP-PCR as a rapid, precise, and genetically based procedure to identify *A. pleuropneumoniae* serotypes. The technique has several advantages over current methods of serotyping this organism.

With AP-PCR, genomic DNA provides information for amplification and subsequent serotype determination. This is of special interest when working with *A. pleuropneumoniae*, because antisera to whole cells have been found to cross-react with antigens of heterologous serotypes (14). AP-PCR does not utilize antisera; therefore, factors such as cross-reacting antibodies do not confound results.

Serotyping also relies heavily on antigenic determinants of the isolates rather than their genetic relatedness. These antigenic determinants are subject to change, however, either spontaneously or with passage of time through antigenic drift. AP-PCR amplifies arbitrarily defined segments of genomic DNA, which may or may not encode virulence or antigenic determinants. Therefore, although genetic relatedness of organisms or serotypes is not distinguished by AP-PCR, because of the clonal nature of *A. pleuropneumoniae* (11) and the small percentage of total genomic DNA that is amplified between sites of primer annealing with this method (<3 kb of the total genome), the chance that genomic alteration will affect these AP-PCR DNA profiles is greatly

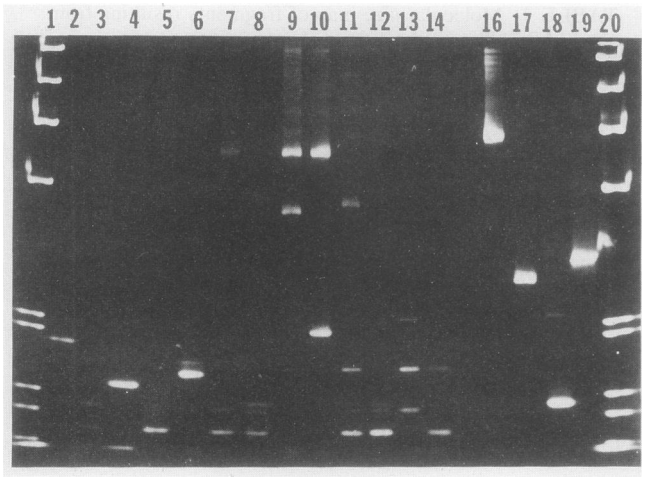

FIG. 3. Comparison of AP-PCR fragments of *A. pleuropneumoniae* and other genera and species with the M13 primer pair. Lanes: 1, 1-kb DNA ladder; 2, Perkin-Elmer Cetus control; 3 to 14, *A. pleuropneumoniae* reference serotypes 1 to 12, respectively; 16, *A. suis*; 17, *E. coli*; 18, *H. parasuis*; 19, *P. multocida*; 20, 1-kb DNA ladder. Amplified products were resolved by electrophoresis on 6% nondenaturing polyacrylamide gel and stained with ethidium bromide. The size marker is the 1-kb DNA ladder from Bethesda Research Laboratories-GIBCO.

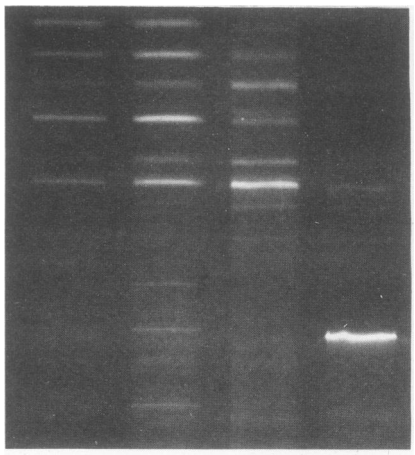

FIG. 4. Comparison of nontypeable field strains of *A. pleuropneumoniae* with T3-T7 primers. DNA preparation was made as described in the text. Amplification was performed in a Perkin-Elmer Cetus 9600 tempcycler programmed for 2 cycles of 1.15 min at 94°C, 1.15 min at 40°C, and 1.15 min at 72°C, followed by 35 cycles of 0.5 min at 94°C, 0.5 min at 60°C, and 0.5 min at 72°C, using the fastest available ramp times between temperatures.

decreased. This is exemplified in a report by Musser et al. (11), which indicates that chromosomal recombination among isolates is very infrequent in *A. pleuropneumoniae*. Using a technique known as multilocus enzyme electrophoresis which detects allelic variation in structural genes, those investigators determined that isolates of the same serotype and outer membrane protein profile were frequently the same or a closely related electrophoretic type.

The hallmark of AP-PCR is its ability to provide highly specific genomic fingerprints without prior knowledge of DNA sequence (31, 32). At a low-stringency annealing temperature, an arbitrarily chosen primer sequence of approximately 20 bases will anneal to several sequences of the genome with a variety of mismatches. The sequences that lie between these sites of annealing, if of proper size constraints, will be amplifiable by PCR. Efficiency of primer annealing and of extension of these interim sites aids in determining the extent to which any of these sequences amplify. By utilizing two cycles of low-stringency annealing and many cycles of high-stringency annealing, reproducible patterns of PCR-amplified genomic fragments can be evaluated readily (31, 32).

Distinctive patterns obtained from amplification of *A. pleuropneumoniae* confirm that AP-PCR allows differentiation between various strains and that the resulting DNA profile can distinguish currently recognized serotypes. However, there is no evidence that a relationship exists between serotype and any specific DNA fragment, nor is that to be expected with this technique.

It is important to note that pure colonies of *A. pleuropneumoniae* must be obtained before DNA amplification by AP-PCR, because DNA from contaminating organisms will dramatically change the profile. Plasmid contamination of genomic DNA of field isolates could also change these profiles. Plasmid DNA would need to be removed by cesium chloride centrifugation, if changes in the amplification profile resulted in an indistinguishable serotype. However, it would be more likely for an extra amplification fragment to be generated from the plasmid DNA, rather than for genomic fragments to change in size. Additionally, amplification fragments from plasmid DNA could potentially serve as identification markers when this technique is used for epidemiological purposes.

Sirois et al. (30) described the use of PCR to specifically detect *A. pleuropneumoniae*, using a primer set derived from sequence data produced by recombinant DNA techniques. Although specific for *A. pleuropneumoniae*, this primer set is not specific for any of the serotypes of *A. pleuropneumoniae* and, therefore, is of use only in the identification of the organism. We feel that standardized AP-PCR holds promise as a rapid serotype-specific identification method for diagnostic and epidemiological investigations of porcine pleuropneumonia outbreaks. Because of the wide availability of the primers that we evaluated and their ability to specifically identify currently recognized serotypes of *A. pleuropneumoniae*, we propose to follow the recommendations of Welsh and McClelland (31) that these primers become standard for *A. pleuropneumoniae* diagnostic PCR serotyping. Size markers for amplified DNA fragments from each serotype need to be determined by each individual laboratory, taking equipment, buffers, and protocols used into account.

For most isolates, a single primer or primer set will be needed to definitively identify a specific serotype. Closely related serotypes, such as serotypes 9 and 1, may need to be compared by amplifying DNA with several primers either combined or used individually. Serotypes 3, 6, and 8 and serotypes 4 and 7, which are serologically related, are easily differentiated with the M13 primers. Ultimately, serotype-specific primers will provide a definitive means of identifying *A. pleuropneumoniae* serotypes under conditions of high stringency.

Within a few hours from the time a bacterial colony is selected, PCR-amplified DNA profiles are available for comparison with serotype-specific patterns previously determined in the laboratory. DNA from nontypeable and cross-reactive *A. pleuropneumoniae* isolates can be amplified by this technique and easily identified. However, profiles that

do not match any currently recognized serotypes should be examined for the presence of plasmid DNA in the organism.

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