Arbitrarily Primed PCR Analysis of *Mycoplasma hyopneumoniae* Field Isolates Demonstrates Genetic Heterogeneity

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***Mycoplasma hyopneumoniae* is the primary agent of mycoplasmal pneumonia in swine. In this study we performed an arbitrarily primed PCR (AP-PCR) analysis, in which low-stringency amplification with a single primer was used, to investigate genetic variability in *M. hyopneumoniae* strains and field isolates. We performed preliminary experiments to examine the efficacy of 40 different 10-mer oligonucleotides for priming an AP-PCR with *M. hyopneumoniae* J^T (T = type strain) chromosomal DNA. On the basis of our results, we selected primers OPA-3, OPA-17, and OPB-10 for use in an analysis performed with 23 field isolates. The most informative results were obtained with primer OPA-3. A total of 21 of 23 clinical isolates produced multiband patterns with this primer, while 2 isolates failed to produce any detectable bands. Our data show that *M. hyopneumoniae* is genetically diverse and that *M. hyopneumoniae* strains can be divided into at least six epidemiological subgroups on the basis of AP-PCR results.**

The mycoplasmas are wall-less prokaryotes that belong to the class *Mollicutes* and are believed to be the smallest organisms that are capable of self-replication. The genome sizes of mycoplasmas vary from 600 to 1,100 kb, and the chromosomal DNA G+C content of most strains is extremely low (about 26 mol%). Members of the genus *Mycoplasma* are isolated from animal hosts, where they may be commensal or pathogenic. *Mycoplasma hyopneumoniae* is the primary agent of mycoplasmal rhinitis and pneumonia in swine (16). This disease is a worldwide problem that results in large economic losses. There is also increasing evidence that *M. hyopneumoniae* has a predisposing influence on other infections (16). Limited information concerning genetic differences among the strains of *M. hyopneumoniae* is available, and consequently no data relating genetic differences to virulence have been published (7). An understanding of genetic variability in *M. hyopneumoniae* is important for studies of the taxonomy, epidemiology, and pathogenicity of this species.

As a first step in developing a genetic typing scheme, arbitrarily primed PCR (AP-PCR), which is sometimes referred to as random amplified polymorphic DNA analysis, was used to determine the extent of genetic variability in *M. hyopneumoniae*. The use of PCR to "type" clinical isolates is becoming commonplace because of the sensitivity and speed of this method (10, 19, 20). This technique has also been applied to mycoplasma species by targeting species-specific DNA sequences (2, 4, 9, 11–13). AP-PCR also allows workers to assess genomic variability in different field isolates (1, 8, 21). Because this method requires less than 50 ng of DNA, it could be particularly important in studies of *M. hyopneumoniae*, a fastidious mycoplasma species which grows slowly and requires expensive specialized media.

M. hyopneumoniae J^T (= ATCC 25934^T) (T = type strain) and all of the field isolates of *M. hyopneumoniae* which we used (Table 1) were obtained from R. F. Ross, Veterinary Medical Research Institute, Iowa State University. All of the field iso-

lates were obtained from clinical samples from diseased swine submitted to the Iowa State University Veterinary Diagnostic Laboratory. These isolates were obtained from different swine herds whose members had mycoplasmal pneumonia symptoms; the herds were located throughout the Midwest (in Iowa, Indiana, and Kentucky). Each isolate was identified by using a fluorescence antibody test (R. F. Ross, Iowa State University), a metabolic inhibition test (6) and a *M. hyopneumoniae*-specific PCR (2). All mycoplasmas were cultivated in Friis medium supplemented with 20% acid-adjusted swine serum and 5% fresh yeast extract for 48 to 72 h at 37°C until the mid-log phase.

To prepare chromosomal DNA, cells from a 10-ml culture were harvested by centrifugation at 20,000 × g for 15 min at 4°C, washed with phosphate-buffered saline (0.14 M NaCl, 0.01 M sodium phosphate; pH 7.3), and resuspended in 100 µl of TE buffer (0.01 M Tris, 0.001 M EDTA; pH 8.0). Then 100 µl of 1% sodium dodecyl sulfate and proteinase K (final concentration, 400 µg/ml; Sigma Chemical Co., St. Louis, Mo.) was added, and the mixture was incubated for 1 h at 37°C. A 10-µl portion of glass milk (Bio 101, Inc., La Jolla, Calif.) was added, and the suspension was incubated for 5 min at 0°C. The glass milk-DNA precipitate was washed three times with New Wash buffer (Bio 101, Inc.) without shaking, and the DNA was eluted with 50 µl of TE buffer at 46°C for 5 min. The DNA concentration of each sample was determined by fluorescence by using a model TKO 100 minifluorometer (Bio-Rad Laboratories, Richmond, Calif.). To verify that the DNA was intact, aliquots were electrophoresed on a Tris-borate–0.7% agarose gel, and the gel was stained with ethidium bromide.

Our initial experiments revealed that the presence of contaminating RNA in DNA preparations resulted in nonreproducible AP-PCR data (data not shown). In order to increase reproducibility and ensure high-quality DNA preparations, chromosomal DNA was isolated by using glass milk and was eluted with TE buffer. No RNA was observed in the resulting DNA preparations. Each DNA preparation was then analyzed by performing a *M. hyopneumoniae*-specific PCR (2). All preparations yielded the expected single 456-bp fragment when the PCR primer pair described previously (2) was used (data not shown).

Two primer kits containing a total of 40 10-mer oligonucleotide primers were obtained from Operon Technologies, Inc., Alameda, Calif. To screen for positive reactions, chromosomal

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TABLE 1. Strains and field isolates of *M. hyopneumoniae* used in this study and the reactivities of these strains with primers OPA-3, OPB-10, and OPA-17

Group	Strain	Source of original culture ^a	Year received ^b	Location ^c	Reactivity with:											Primer OPB-10	Primer OPA-17
					Primer OPA-3												
					2.5-kb band	1.8-kb band	1.6-kb band	1.4-kb band	1.3-kb band	1.0-kb band	0.8-kb band	0.7-kb band	0.6-kb band	0.5-kb band			
I	10954	VMRI	1983	Iowa	—	—	+	—	—	—	+	—	+	—	—	—	
	P-13129-5	Armstrong	1981	Indiana	—	—	+	—	—	—	+	—	+	—	—	—	
	P-13129-6	Armstrong	1981	Indiana	—	—	+	—	—	—	+	—	+	—	+	—	
	P-13129-9	Armstrong	1981	Indiana	—	—	+	—	—	—	+	—	+	—	+	—	
	P-11318	Armstrong	1982	Indiana	—	+	+	+	—	—	+	—	+	—	+	—	
II	31-9	VMRI	1989	Kentucky	—	+	+	+	+	—	+	+	+	—	+	—	
	P-1814-5	Armstrong	1981	Indiana	—	+	+	+	+	—	+	+	+	—	+	+	
	P-1814-10	Armstrong	1981	Indiana	—	+	+	+	+	—	+	—	+	—	+	—	
	P-5782	Armstrong	1980	Indiana	—	+	+	+	+	—	+	—	+	—	+	—	
III	11928	VMRI	1983	Iowa	—	+	+	+	+	—	+	—	+	—	—	+	
	P-6053-4	Armstrong	1981	Indiana	—	—	+	+	+	+	+	—	+	—	—	+	
	P-6053-3	Armstrong	1981	Indiana	—	+	+	+	+	—	+	—	+	—	—	—	
	P-6053-2	Armstrong	1981	Indiana	—	+	+	+	+	—	+	—	+	—	—	—	
IV	18-14	VMRI	1989	Kentucky	—	+	—	+	+	—	—	+	—	—	+	—	
	3-14	VMRI	1989	Kentucky	—	+	—	+	+	—	—	+	—	—	+	—	
	16-14	VMRI	1989	Kentucky	—	+	—	+	+	—	—	+	—	—	—	—	
	4-14	VMRI	1989	Kentucky	+	+	—	+	+	—	—	+	—	—	—	—	
	P-5298-1	Armstrong	1982	Indiana	—	+	+	+	+	—	—	+	—	—	—	—	
	J ^T (= ATCC 25934 ^T)				—	+	—	+	+	+	—	—	—	—	+	+	
	10986	VMRI	1983	Iowa	—	+	—	+	+	—	—	—	—	—	—	—	
V	9275	VMRI	1983	Iowa	—	+	—	+	+	—	+	—	—	+	—	—	
	10731	VMRI	1983	Iowa	—	+	—	+	—	—	+	—	—	—	—	—	
VI	11684C	VMRI	1983	Iowa	—	—	—	—	—	—	—	—	—	—	+	—	
	37-9	VMRI	1989	Kentucky	—	—	—	—	—	—	—	—	—	—	—	—	

^a VMRI, Veterinary Medical Research Institute, Iowa State University, Ames; Armstrong, C. Armstrong, Purdue University Veterinary Diagnostic Laboratory, West Lafayette, Ind.

^b The year the isolate was received at the Veterinary Medical Research Institute; the actual year of isolation is unknown in most cases.

^c Geographical location where the strain was isolated.

DNA from strain J^T was amplified in a 30- μ l reaction mixture which contained 30 ng of mycoplasma genomic DNA, 2 mM MgCl₂, 5 pmol of primer, 100 μ M dATP (Pharmacia Fine Chemicals), 100 μ M dCTP (Pharmacia), 100 μ M dGTP (Pharmacia), 100 μ M dTTP (Pharmacia), 1 $\times$ AmpliTaq buffer, and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). The reaction conditions were then optimized for reproducibility with primer OPA-3 (3 mM MgCl₂, 20 pmol of primer, and each nucleotide at a concentration of 200 μ M). The program used for all PCR was a 45-cycle program, with each cycle consisting of 1 min at 92°C, 1 min at 36°C, and 2 min at 72°C. An Ericomp Twin Block thermal cycler was used for amplification. A 15- μ l portion of amplification reaction products was analyzed by electrophoresis in Tris-borate–0.7% agarose–0.4% Synergel (Diversified Biotech, Boston, Mass.) gels, the bands were detected by staining with ethidium bromide, and the image was digitized by using a model 4900 high-performance charge-coupled device (CCD) camera (Cohu, Inc., San Diego, Calif.).

Only 6 of the 40 primers used (primers OPA-3 [AGTCAGC CAC], OPA-17 [GACCGCTTGT], OPB-8 [GTCCACACGG], OPB-10 [CTGCTGGGAC], OPB-14 [TCCGCTCTGG], and OPB-18 [CCACAGCAGT]) gave positive results under the PCR conditions used. Primers OPB-8 and OPB-18 produced a single band with strain J^T DNA. Primers OPA-17 and OPB-14 produced strong bands, and primers OPB-10 and OPA-3 pro-

duced multiple bands. Primers OPA-3, OPA-17, and OPB-10 were used in subsequent studies with field isolates of *M. hyopneumoniae*.

The results of one set of AP-PCR with primer OPA-3 are shown in Fig. 1. A total of 21 of 23 *M. pneumoniae* field isolates produced a multiple-band pattern, while 2 isolates, strains 11684C and 37-9, failed to produce any signal. The latter result can be interpreted as the loss of specific sites for primer binding in the chromosomal DNAs of these isolates since these DNAs gave the appropriate bands when they were examined by using the species-specific PCR described previously (2) (data not shown).

The sizes of the primer OPA-3-generated fragments were determined from digitized images by using the GelReader 2.0.5 software (National Center for Supercomputing Applications, University of Illinois at Urbana-Champaign). On the basis of these results, each strain was visually coded for the presence or absence of 10 different bands plus reactivity to primers OPA-17 and OPB-10 (Table 1). Although there were variations in the intensities of the bands obtained with primer OPA-3, these variations were not included in the analysis. The data for 24 strains (strain J^T and 23 independent field isolates) were then analyzed with the Phylogenetic Analysis Using Parsimony package (PAUP version 3.1.1) (17). We also included in this analysis the AP-PCR results obtained by using primers

FIG. 1. One set of AP-PCR for different *M. hyopneumoniae* field isolates obtained by using primer OPA-3. AP-PCR were performed in mixtures containing 3 mM $MgCl_2$ and 20 pmol of primer and were analyzed as described in the text. The lanes contain AP-PCR products obtained with the DNAs of selected strains. The lane on the left contained a kilobase ladder molecular weight standard (Bethesda Research Laboratories), and the numbers on the left indicate fragment sizes (in kilobases). The figure is a composite of two gels that were electrophoresed simultaneously; all of the samples used were prepared on the same day under identical conditions. The gels were digitized as described in the text by using a Macintosh IIfx computer equipped with a video board (Scion Corp., Frederick, Md.). The resulting TIFF files were cropped and assembled in Adobe Photoshop and were labeled in Aldus FreeHand.

OPA-17 and OPB-10 (Table 1). Heuristic analyses were performed with stepwise random addition by using branch swapping with tree bisection and reconnection. A consensus tree was then generated from the equally most parsimonious trees by using the 50% majority rule (Fig. 2).

The *M. hyopneumoniae* isolates belonged to six separate groups (Fig. 2). Group I included two strains that failed to react with primer OPA-3. One of these strains, strain 37-9, failed to react with any of the PCR primers used, although it did react with the *M. hyopneumoniae*-specific primer pair (data not shown). Our results also revealed similarities of band patterns within geographical regions. For instance, four of the five isolates in group II were isolated from diseased swine from Indiana. As far as we could determine, each of these isolates was from a different swine farm within the state. The members of each of the four larger groups, groups II through V, exhibited similar geographical distributions (Table 1). Even within a single geographical location, however, there were differences in the band patterns and the reactivities as determined by AP-PCR with the three oligomers (Table 1). The results of our analyses of the data by state are shown in Fig. 3. In each of these analyses 100 replicates were examined. Even grouping by state clearly revealed variability in the band patterns. In contrast, *Mycoplasma pneumoniae* appears to be highly clonal in nature when it is examined by AP-PCR (18).

Most of the information about *M. hyopneumoniae* which we have has been obtained by analyzing type strain J (= ATCC 25934), which is thought to be indistinguishable from field strains on the basis of growth characteristics, metabolic inhibition with type-specific antisera, and immunological tests (14, 15). Chromosomal heterogeneity was also observed in *M. hyopneumoniae* field strains when a pulsed-field gel electrophoresis analysis of restriction enzyme digests was performed (7).

FIG. 2. Majority rule consensus grouping of 24 *M. hyopneumoniae* strains. The AP-PCR band patterns of 24 *M. hyopneumoniae* strains were analyzed by performing a heuristic analysis with random stepwise addition of 100 replicates as described in the text. A total of 70 equally parsimonious trees were generated, and a consensus tree was generated by using the 50% majority rule. The numbers are the percentages of trees containing particular branch points.

FIG. 3. Analysis of AP-PCR data by state. The data were sorted by state, and each resulting group was analyzed individually as described in the text. We used random stepwise addition in this analysis. Majority rule consensus trees were constructed for the strains from Indiana (A) and Iowa (B). Only one parsimonious tree was generated with the strains from Kentucky (C). The numbers are the percentages of trees containing particular branch points.

Antigen preparations from broth-grown *M. hyopneumoniae* cultures contain large amounts of contaminating broth proteins, a common problem in studies with mycoplasmas (5, 22) which complicates protein and immunological analyses. Consequently, the differences in virulence between avirulent strain J^T and field isolates are not easily explained.

Our data demonstrated that there is significant genetic heterogeneity in *M. hyopneumoniae* field isolates. The true extent of the heterogeneity in *M. hyopneumoniae* is not known since single base pair changes in the DNA sequence could result in the loss or acquisition of single bands. Analyses of additional field isolates from other geographical regions may reveal additional groups, but this technique is only one measure of genetic relatedness and its resolving power is limited. Our results must still be interpreted cautiously since two dissimilar PCR products may resolve together and variability in a region may not be revealed by agarose gel electrophoresis.

Since not all of the strains which we studied have been examined for disease potential by using a swine challenge model, it is not possible to correlate genetic heterogeneity with virulence with certainty. However, it can be safely assumed that each of the field isolates is pathogenic since the strains were isolated from diseased swine that exhibited mycoplasmal pneumonia symptoms.

It is thought that mycoplasmas evolve rapidly, and our AP-PCR results may simply be another reflection of this evolutionary process. We do not know whether the variation identified in this study was due to genomic instability or whether substantial evolution has occurred in some *M. hyopneumoniae* populations because of selective pressures. This could be ascertained by studying a single well-defined isolate as it progresses through a swine herd to determine how rapidly genomic variation occurs in this species. Chromosomal rearrangements have been identified previously in the mycoplasmas (3), which may account for some of the variability observed in the field isolates. AP-PCR may be useful for studying genomic variation in the field and thereby may increase our knowledge concerning mycoplasmal pathogenesis. This technique can also be performed easily and could form the basis of a useful typing scheme for *M. hyopneumoniae*.

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