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

SERGEY ARTIUSHIN† AND F. CHRIS MINION*
Veterinary Medical Research Institute, Iowa State University, Ames, Iowa 50011

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 J1 (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.
DNA from strain J\textsuperscript{+} was amplified in a 30-\textmu l reaction mixture which contained 30 ng of mycoplasmal genomic DNA, 2 mM MgCl\textsubscript{2}, 5 pmol of primer, 100 \mu M dATP (Pharmacia Fine Chemicals), 100 \mu M dCTP (Pharmacia), 100 \mu M dGTP (Pharmacia), 100 \mu M dTTP (Pharmacia), 1X 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 MgCl\textsubscript{2}, 20 pmol of primer, and each nucleotide at a concentration of 200 pM). 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.

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 primer J\textsuperscript{+} (\textasciitilde ATCC 29394\textsuperscript{a}).

The results of one set of AP-PCR with primer OPA-3 are shown in Fig. 1. A total of 21 of 23 \textit{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\textsuperscript{+} 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 produced multiple bands. Primers OPA-3, OPA-17, and OPB-10 were used in subsequent studies with field isolates of \textit{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 \textit{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).
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 MgCl₂ 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 IIci 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).
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 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 have 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*.

We thank Barbara Zimmerman-Erickson and R. F. Ross for supplying the original cultures used in this study. We also thank Mel Duvall for his assistance in interpreting the parsimony analysis.

This work was supported by Cooperative State Research Service grant 91-37204-6488 from the United States Department of Agriculture. F.C.M. is the recipient of U.S. Public Health Service Research Career Development Award 1K04 AI-01021 from the National Institute of Allergy and Infectious Diseases.

REFERENCES


