J. Microbiol. Biotechnol. (2009), **19**(3), 265–270
doi: 10.4014/jmb.0806.347
First published online 10 October 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY
© The Korean Society for Microbiology and Biotechnology

Oligonucleotide Array-based Detection and Genotyping of Mollicutes (*Acholeplasma, Mycoplasma*, and *Ureaplasma*)

Jang, Hyunjung[1], Hyomyeung Kim[1], Byeongchul Kang[3], Cheolmin Kim[1,2], and Heekyung Park[4]*

[1]*Institute for Genomic Medicine, GeneIn Co., Ltd, Busan 617-030, Korea*
[2]*Department of Biochemistry, Pusan National University Medical School, Busan 602-739, Korea*
[3]*Institute of Supportive Bioinformatics, Insilicogen Inc., Suwon 441-813, Korea*
[4]*PANAGENE Inc., 816 Tamnip-dong, Yuseong-gu, Daejeon 305-510, Korea*

Received: June 2, 2008 / Accepted: July 24, 2008

An oligonucleotide array was developed to detect and genotype mollicutes based on the internal transcribed spacer (ITS) sequence. The results of the assay were compared with those of a PCR-RFLP assay. The proposed oligonucleotide array containing 5 genus- and 23 species-specific probes was able to detect Mycoplasma species, including *M. penetrans* and *M. spermatophilum*, that were not detected by the PCR-RFLP assay. Therefore, the results demonstrated that the proposed oligonucleotide array was effective for the detection and discrimination of 23 species, including an acholeplasma, 21 mycoplasmas, and a ureaplasma, and showed promise as a countermeasure to ensure that biological products are safe and of good quality.

Keywords: Oligonucleotide array, mollicute, internal transcribed spacer, genotyping

The contamination of biological materials by mollicutes (including *Acholeplasma, Mycoplasma*, and *Ureaplasmas*) can lead to unreliable experimental results and unsafe biological products. Mycoplasmas are phenotypically distinguished from other bacteria by their minute size and total lack of a cell wall. The methods for detecting pathogenic or contaminant mycoplasmas include microbiological cultures, immunological assays, DNA staining techniques, nucleic acid hybridization, and PCRs [4, 11]. Various laboratories have already developed species-specific PCR assays to detect mycoplasmas [12]. However, PCR assays can only detect a single or limited number of mollicute species [5, 10]. Accordingly, this study analyzed a phylogeny tree of the ITS sequences of mollicutes and developed an oligonucleotide array that can genotype 23 mollicutes (an acholeplasma, 21 mycoplasmas, and a ureaplasma).

*Corresponding author
Phone: +82-42-936-9298; Fax: +82-42-861-9297;
E-mail: heekyung.park@panagene.com

Bacterial Strains and Preparation of Genomic DNAs

Acholeplasma laidlawii ATCC 25937, *Mycoplasma arginini* ATCC 23838, *Mycoplasma arthritidis* ATCC 19611, *Mycoplasma bovis* ATCC 27368, *Mycoplasma cloacale* ATCC 35276, *Mycoplasma falconis* ATCC 51372, *Mycoplasma faucium* ATCC 25293, *Mycoplasma fermentans* ATCC 19989, *Mycoplasma genitalium* ATCC 33530, *Mycoplasma hominis* ATCC 23114, *Mycoplasma hyorhinis* ATCC 17981, *Mycoplasma hyosynoviae* ATCC 25591, *Mycoplasma neurolyticum* ATCC 19988, *Mycoplasma opalescens* ATCC 27921, *Mycoplasma orale* ATCC 23714, *Mycoplasma penetrans* ATCC 55252, *Mycoplasma pirum* ATCC 25960, *Mycoplasma pneumoniae* ATCC 15531, *Mycoplasma primatum* ATCC 15497, *Mycoplasma salivarium* ATCC 23064, *Mycoplasma spermatophilum* ATCC 49695, *Mycoplasma synoviae* ATCC 25204, and *Ureaplasma urealyticum* ATCC 27618 were all purchased from the American Type Culture Collection (ATCC), whereas the other bacteria, *Bacillus subtilis* KCTC 1103, *Lactobacillus casei* KCTC 2180, *Clostridium pasteurianum* KCTC 1674, *Escherichia coli* KCTC 1042, and *Enterococcus faecalis* KCTC 2011, were obtained from the Korean Collection for Type Cultures (KCTC). The genomic DNAs were extracted using an InstaGene matrix kit (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.) according to the manufacturer's protocol.

PCR and Sequencing of ITS Regions

The primers used for the amplification of the ITS region of the mycoplasmas (F2 and R2) and acholeplasmas (M78 and R34) have already been described [10]. The PCR mixture (50 μl) contained 1 U of *Taq* DNA polymerase, 1× Qiagen PCR buffer (Qiagen Inc., Valencia, CA, U.S.A.), and 20 pmol of each primer. The PCR conditions included 4 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; plus a further 10 min at 72°C. The PCR products were sequenced after subcloning using a TA Cloning Kit (Promega, Madison, WI, U.S.A.), and compared with existing

sequences in the GenBank database. The ITSs of *M. bovis, M. cloacale, M. falconis, M. faucium, M. spermatophilum,* and *M. synoviae* were newly sequenced in this study.

Phylogenetic Analysis

The distances for each ITS were calculated using the PHYLIP package [2] and Kimura 2 parameter [4]. The phylogenetic trees were also constructed with the PHYLIP package using the neighbor-joining method [8]. The significance of the resulting tree topologies was tested by performing a bootstrap analysis with resampling 1,000 times.

Oligonucleotide Array for Genotyping of Mollicutes

Genus- and species-specific probes were designed from the conserved and polymorphic regions of the ITSs for *Acholeplasma, Mycoplasma*, and *Ureaplasma*, respectively. The specificity of the probes was evaluated by testing using mollicutes and closely related bacteria (*B. subtilis, Lb. casei, Cl. pasteurianum, E. coli,* and *En. faecalis*).

The fabrication, hybridization, and scanning processes of the oligonucleotide array have been described previously [3, 6]. A schematic showing the layouts of the oligonucleotide probes is shown in Figs. 2Y and 2Z.

PCR-Restriction Enzyme Analysis (PRA)

The unique restriction patterns predicted by the NEB cutter, version 2.0 program (http://www.neb.com/rebase), were verified using the PCR products amplified with the F2 and R2 primers for the restriction enzyme analysis of the mollicutes. Differentiation of the mollicute species was accomplished by digestion with VspI (MBI Fermentas, Hanover, MD, U.S.A.), SspI and HindIII (New England BioLabs, Beverly, MA, U.S.A.), ClaI, DraI, and HinfI (DCC-BIONET, Korea), and the digested patterns determined by agarose gel electrophoresis.

Phylogenetic Relationships of Mycoplasmas Based on ITS Sequences

An acholeplasma, 21 mycoplasmas, and a ureaplasma were clustered in 5 groups based on the ITS sequences (Fig. 1). The overall ITS sequence similarity ranged from 32.0% to 99.0% (data not shown). The lowest similarity was 32.0% between *M. hyorhinis* and *M. pirum*, whereas the highest similarity was 99% between *M. arthritidis* and *M. faucium* (data not shown). Therefore, these results indicated that the phylogenetic analysis using the ITS sequences was capable of distinguishing between an acholeplasma, 21 mycoplasmas, and an ureaplasma, with the exception of *M. arthritidis* and *M. faucium*.

Genotyping of Mycoplasmas Using Oligonucletide Array: Specificity and Sensitivity

The ITS sequences of mollicutes contain both conserved and polymorphic regions. Thus, the conserved regions in the ITS sequences of *Acholeplasma* and *Mycoplasma* were used to design several genus-specific probes. First, the optimal genus-specific probes were selected for 5 groups based on the ITS sequences. The MP-CP probe was hybridized with the mycoplasmas in groups 1 and 3. The MP-CB probe was hybridized with the mycoplasmas in group 2. The MP-CE probe was hybridized with the mycoplasmas in groups 4 and 5. In addition, since there was no single genus-specific probe to

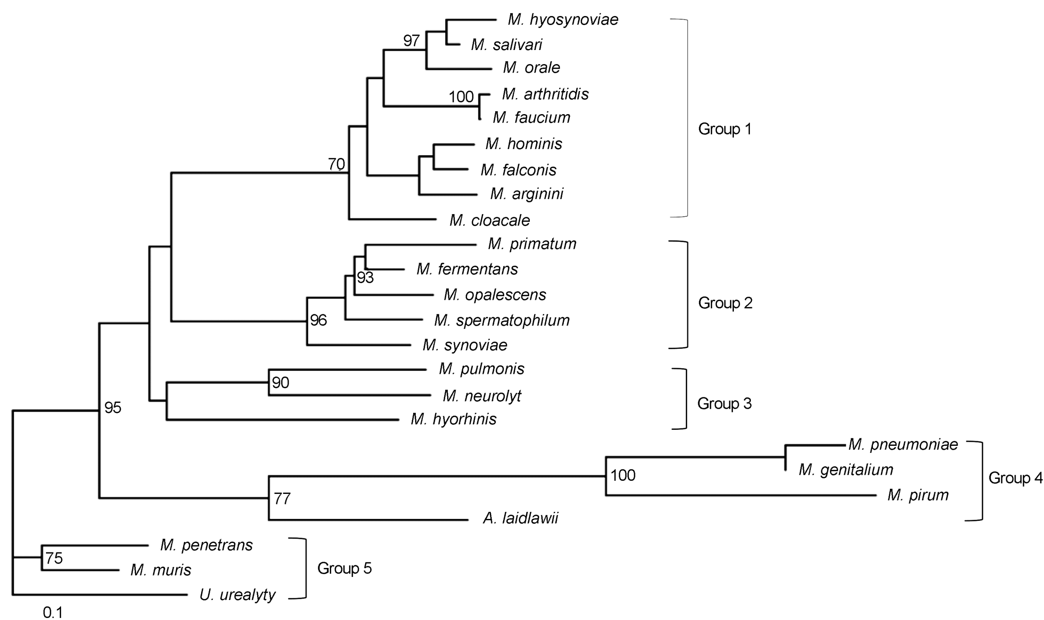

Fig. 1. Phylogenic tree derived from ITS region sequences of mycoplasmas.
The numbers on the tree are the percentages of occurrence in 1,000 bootstrapped trees; only values greater than 70% are shown.

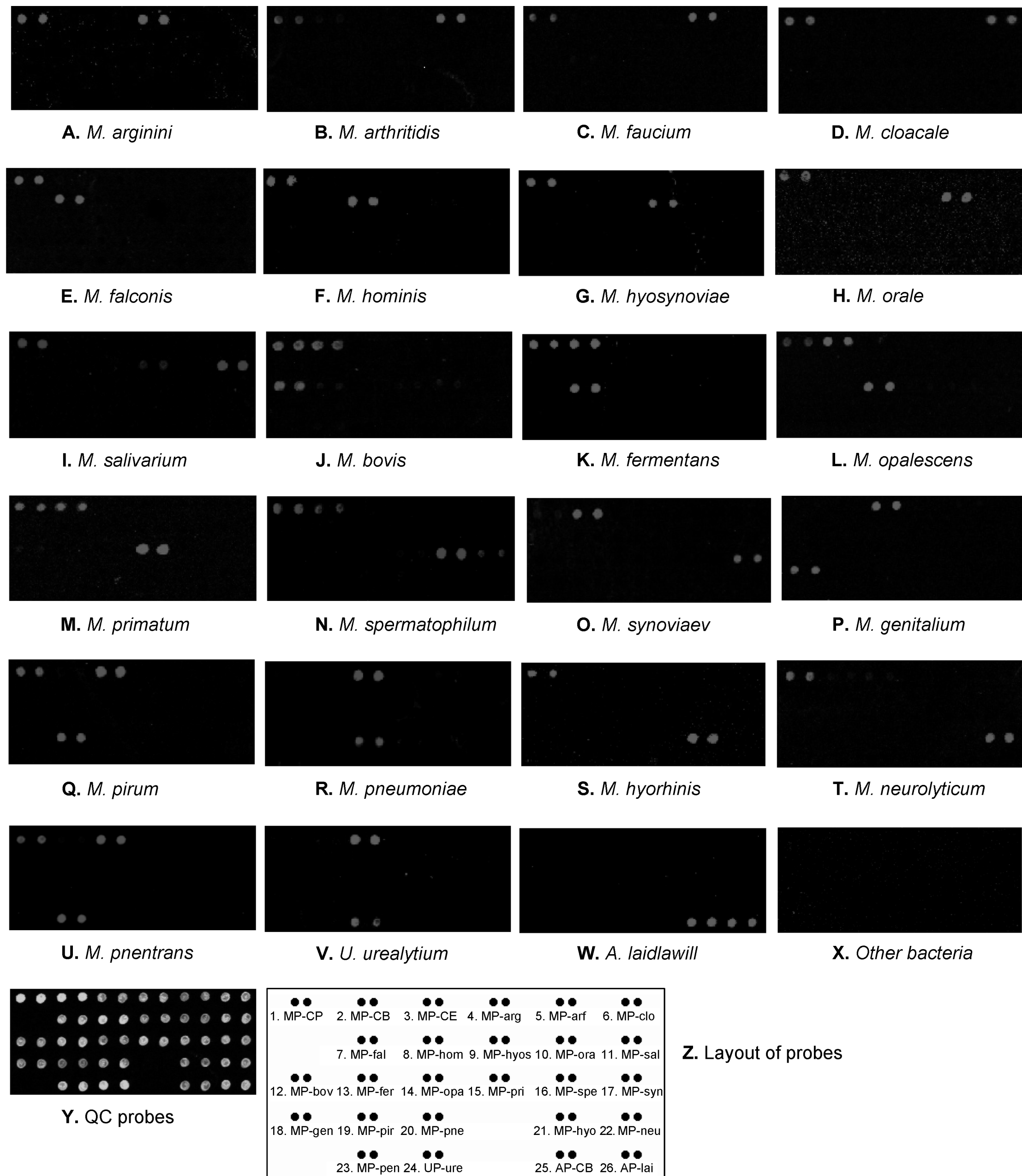

Fig. 2. Hybridization results for mycoplasma species when using an oligonucleotide array.

A to **W**. Each species of mycoplasma exhibited a unique pattern of reactivity to each set of probes. **X**. Hybridization results for other pathogenic bacteria. **Y**. Image of QC probe before hybridization. **Z**. Layout of probes in the oligonucleotide array. The probes were patterned in duplicate. The probe names are as explained in Table 1.

hybridize all the mycoplasmas, a combination of the four probes (MP-CP and MP-CB or MP-CP and MP-CE) was used to detect the mycoplasmas at the genus level. Finally, the AP-CB probe was hybridized with the acholeplasma.

The presence of several polymorphic regions within the ITS sequences enabled the design of individual species-specific probes. The genus- and species-specific probes only detected their target, and there was no cross-hybridization with other bacteria (Fig. 2). The oligonucleotide array allowed for the differentiation of an acholeplasma, 21 mycoplasmas, and a ureaplasma, yet was unable to differentiate *M. arthritidis* and *M. faucium*, owing to the 99% similarity of their respective ITS sequences. The oligonucleotide array also allowed for the identification of a number of mycoplasma species in one reaction. The genus-specific probes showed a unique hybridization pattern with mycoplasmas (Figs. 2A to 2W) and a mismatch with other bacteria containing *B. subtilis*, *Lb. casei*, *Cl. pasteurianum*, *E. coli*, and *En. faecalis*,

Table 1. Probes designed in this study.

Strain	Probe	Sequence	Position[c]
Acholeplasma[a]	AP-CB	5'-T_{15}-AGTCTTTGAAAAGTAGATAAA-3'	134–154
	MP-CP	5'- T_{15}-RWTCTTTVAAAACTRRATWN-3'	74–93[d]
Mycoplasma[b]	MP-CB	5'- T_{15}-GGKYAATTTGTTTWGAT-3'	28–44[d]
	MP-CE	5'- T_{15}-AATAAGTTACTAAGGGCTTAT-3'	201–221[e]
M. arginini	MP-arg	5'- T_{15}-AGATTATATCATACAATAGA-3'	145–164
M. arthritidis-faucium	MP-arf	5'- T_{15}-GAATACAAAATCAATACAATA-3'	146–166[f]
M. cloacale	MP-clo	5'-T_{15}-AGTACAATTCTCACTGTTATG-3'	11–31
M. falconis	MP-fal	5'-T_{15}-GAGTACAACTTCTGTTATG-3'	10–28
M. hominis	MP-hom	5'- T_{15}-ATTTATCTCTCGGTTCTTT-3'	60–78
M. hyosynoviae	MP-hyos	5'-T_{15}-CTAGACTAAAGTTAATGGTAC-3'	40–60
M. orale	MP-ora	5'-T_{15}-CATAAATAGTTAATGGCTCA-3'	36–55
M. salivarium	MP-sal	5'-T_{15}-TAATGGATTTAATTTTCGTG-3'	46–65
M. bovis	MP-bov	5'-T_{15}-GTCATGGCTTTTATTAATAGG-3'	83–103
M. fermentans	MP-fer	5'-T_{15}-TTTTTATGGGTCTAAAGCTTT-3'	93–113
M. opalescens	MP-opa	5'-T_{15}-CATCATAATGTAACCAATAC-3'	270–289
M. primatum	MP-pri	5'-T_{15}-TCATGGGCTTTTAATAGGGTC-3'	90–110
M. spermatophilum	MP-spe	5'-T_{15}-TTCATCGAGATAGTCATTTTA-3'	248–268
M. synoviae	MP-syn	5'-T_{15}-ACCTCTCTTAAAATTGTTCTT-3'	129–149
M. genitalium	MP-gen	5'-T_{15}-CAACTAACACACTTGGTCAGT-3'	83–103
M. pirum	MP-pir	5'-T_{15}-TAGTTCTTTGTGTGAATAACA-3'	222–242
M. pneumoniae	MP-pne	5'-T_{15}-GTAAATTAAACCCAAATCCC-3'	85–104
M. hyorhinis	MP-hyo	5'-T_{15}-CGGAGTACATTAGTCTTAATT-3'	8–28
M. neurolyticum	MP-neu	5'-T_{15}-TAATTTTTTCTTTCTAATTAA-3'	114–134
M. penetrans	MP-pen	5'-T_{15}-AAGAGTAAGTTCTAGGTCG-3'	103–121
U. urealyticum	UP-ure	5'-T_{15}-TAATTTACGTACTAATAAGTG-3'	17–37
A. laidlawii	AP-lai	5'-T_{15}-TTAAAGTAATTTAAGTGTTTC-3'	100–120

[a] *Acholeplasma* genus-specific probe.
[b] *Mycoplasma* genus-specific probe.
[c] Probe position of ITS for *Acholeplasma* and *Mycoplasma* species.
[d] Probe position of *M. arginini.*
[e] Probe position of *M. genitalium*.
[f] Probe position of *M. faucium.*

all of which are taxonomically close to mycoplasmas (Fig. 2X). Each mycoplasma species exhibited a unique hybridization pattern with their own species-specific probe (Fig. 2), allowing each mycoplasma species to be identified with a single probe. The species-specific probes were also able to discriminate the mycoplasmas on the species level under uniform hybridization conditions.

To determine the sensitivity of the PCR-based hybridization assay, a series of 10-fold plasmid dilutions was used, ranging from 29.7 to 0.297 ng/μl per reaction. The sensitivity of the oligonucleotide array was 2.97 pg/μl of DNA per reaction (data not shown).

Genotyping of Mycoplasmas by PCR-Restriction Enzyme Analysis (PRA)

The sizes of the fragments coincided with those predicted on the basis of their sequences (Table 2). Unique restriction patterns were obtained with VspI (data not shown), except for *M. arthritidis* and *M. faucium,* where the restriction patterns were identical. For further verification of the remaining 7 species that could not be identified with VspI, a PRA was also performed with HindIII, ClaI, and SspI. The PRA patterns for *M. arginini* (134 bp and 102 bp) and *M. cloacale* (135 bp and 115 bp) were too similar for discrimination by gel electrophoresis. However, the results of the PCR-RFLP and direct sequencing matched well with those of the oligonucleotide array.

Most of the methods currently available for the detection of mycoplasmas are inadequate in terms of sensitivity and specificity. Therefore, recent efforts have focused on the development of more rapid and accurate detection assays for the species-specific identification of mycoplasmas using sequence-based molecular techniques. Whereas the 16S rDNA identity of mycoplasmas ranges from 62.7% to 98.8%, the ITS sequence identity of mycoplasmas ranges from 32.0% to 99.0%. Thus, a phylogenetic analysis based on ITS sequences

Table 2. Expected lengths of mycoplasmas from the F2/R2 primer pair and restriction fragment size variation of ITS regions in *Mycoplasma* species.

Species	Amplicons size (bp)	Size of restriction fragments (bp)			
		VspI	HindIII	ClaI	SspI DraI
A. laidlawii	590, 340	310, 280, 190, 150			
M. arginini	236	134, 102			
M. arthritidis	280	160, 120			
M. bovis [a]	380	285, 265, 115, 95			
M. cloacale [a]	250	135, 115			
M. falconis [a b]	246	125, 121			
M. faucium [a b]	272	150, 122			
M. fermentans	365	270, 95			
M. genitalium	245	185, 60	241, 124		
M. hominis	236	123, 113			
M. hyorhinis	315	-			
M. hyosynoviae	250	148, 94	-	253, 62	
M. muris	270	-			
M. neurolyticum	380	220, 160	-	-	
M. opalescens [b]	360	206, 154			
M. orale	290	151, 139	244, 116		
M. penetrans [b]	299	-			
M. pirum	323	169, 154			
M. pneumoniae	275	-		-	
M. primatum [b]	348	272, 76	-	-	
M. pulmonis	350	190, 160			
M. salivarium	269	-	-	-	
M. spermatophilum [a]	350	-	-	-	179, 90
M. synoviae [a b]	330	218, 112	-		
U. urealyticum	360	-			

[a] The ITS regions for these six species are not in GenBank, but were obtained by a PCR and sequencing in this study.
[b] The ITS regions for these six species are in GenBank, yet these species are not listed in previous literature [7].
The expected lengths of the restriction fragment size were estimated based on an analysis of the sequences of the PCR amplicon from the F2/R2 primer pair in this study.
- ; No restriction site.

enabled the mollicutes to be clustered into groups and discriminated as species (Fig. 1). Moreover, the size of the amplicons containing conserved and polymorphic regions was 350 to 450 bp, making them appropriate to gain meaningful signals for the hybridization reaction of an oligonucleotide array. Thus, genus- and species-specific probes were designed for the genotyping of mycoplasmas. However, owing to the over 170 species already included in the GenBank database, and their ever increasing number, along with the number of different hosts [9], many probes are needed that can detect more mollicutes. Yet, from a cost perspective, it is not efficient to include species that only contaminate with a low frequency. Thus, to solve both issues, the inclusion of nonspecies-specific probes in the oligonucleotide array allows mycoplasmas to be detected on the genus level by hybridization with a genus-specific probe, thereby including the detection of low-frequency or unknown species of mollicutes. Furthermore, to develop oligonucleotide array-based genotyping, the critical point is the specificity of the genotype-specific probes. Thus, the hybridization-based assay exhibited no cross-reaction, and all the genotype-specific probes were highly discriminating [1].

In conclusion, an ITS sequence-based oligonucleotide array was developed for the detection and discrimination of *Mycoplasma* species using a single hybridization. The proposed oligonucleotide array is able to identify 23 *Mycoplasma* species, with the exception of *M. arthritidis* and *M. faucium*, using a single probe in one hybridization, along with the

detection of unusual or unknown mollicutes in a rapid, easy-to-perform manner and at a low cost. Therefore, the present results demonstrate that an oligonucleotide array is very useful for the rapid identification and accurate discrimination of mycoplasmas, and can be an effective countermeasure to ensure the good quality and safety of biological products.

Acknowledgment

This work was supported by a Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2004-217-C00020).

References

1. Chizhikov, V., M. Wagner, A. Ivshina, Y. Hoshino, A. Z. Kapikian, and K. Chumakov. 2002. Detection and genotyping of human group A rotaviruses by oligonucleotide microarray hybridization. *J. Clin. Microbiol.* **40:** 2398–2407.
2. Felsenstein, J. 1989. PHYLIP - phylogeny inference package. *Cladistics* **5:** 164–166.
3. Jang, H. J., M. Cho, J, Heo, H. Kim, H. Jun, W. Shin, B. Cho, H. Park, and C. Kim. 2004. Oligonucleotide chip for detection of Lamivudine-resistant hepatitis B virus. *J. Clin. Microbiol.* **42:** 4181–4188.
4. Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence. *J. Mol. Evol.* **16:** 111–120.
5. Kong, F., G. James, S. Gordon, A. Zelynski, and G. L. Gilbert. 2001. Species-specific PCR for identification of common contaminant mollicutes in cell culture. *Appl. Environ. Microbiol*. **67:** 3195–3200.
6. Park, H., H. Jang, E. Song, C. L. Chang, M. Lee, S. Jeong, J. Park, B. Kang, and C. Kim. 2005. Detection and genotyping of *Mycobacterium* species from clinical isolates and specimens by oligonucleotide array. *J. Clin. Microbiol.* **43:** 1782–1788.
7. Rottem, S. and M. F. Barile. 1993. Beware of mycoplasmas. *Trends Biotechnol*. **11:** 143–151.
8. Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.
9. Stakenborg, T., J. Vicca, P. Butaye, D. Maes, T. de Baere, R. Verhelst, *et al*. 2005. Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of *Mycoplasma* species. *BMC Infect Dis*. **5:** 46.
10. Tang, J., M. Hu, S. Lee, and R. Roblin. 2000. A polymerase chain reaction based method for detecting *Mycoplasma/Acholeplasma* contaminants in cell culture. *J. Microbiol. Methods* **39:** 121–126.
11. Vora, G. J., C. E. Meador, D. A. Stenger, and J. D. Andreadis. 2004. Nucleic acid amplification strategies for DNA microarray-based pathogen detection. *Appl. Environ. Microbiol.* **70:** 3047–3054.
12. Yoshida, T., S. Maeda, T. Deguchi, and H. Ishiko. 2002. Phylogeny-based rapid identification of mycoplasmas and ureaplasmas from urethritis patients. *J. Clin. Microbiol.* **40:** 105–110.