EJ072200000127

J Microbiol Immunol Infect
2000;33:127-130

Application of representational difference analysis to cloning a *Mycoplasma arthritidis* specific DNA fragment

Chiung-Sui Kuo[1], Hong-Shang Hong[2], Jwo-Farn Chiou[1]

[1]Graduate Institute of Microbiology, College of Medicine, National Taiwan University; [2]Department of Dermatology, Chang Gung Memorial Hospital, Taipei, Taiwan, ROC

Received: September 14, 1999 Revised: September 29, 1999 Accepted: November 9, 1999

Representation difference analysis (RDA) was applied to isolate a *Mycoplasma arthritidis* specific DNA fragment. The DNA fragment obtained was verified to be *M. arthritidis* specific by polymerase chain reaction (PCR) and dot blot hybridization tests. The size of this fragment was 194 bp and the nucleotide sequence was also determined.

Key words: *Mycoplasma arthritidis*, representation difference analysis

Mycoplasma arthritidis and *Mycoplasma pulmonis* are significant causes of infection in colonies of rodents used for experimental and biomedical research [1]. The differential diagnosis of these two infections is difficult because of the similarity of serological, pathological, and clinical syndromes involving these two murine mycoplasma species [2,3]. Recently developed DNA probes for the detection of mycoplasma [4,5] appear to enable differential diagnosis to be performed. However, obtaining these species specific probes is an arduous task when conventional methods are used involving screening the clones of a constructed genomic library, or subtracting restriction enzyme treated genomic DNA fragments with the genomic DNA of other species. Recently, a new technique known as representational difference analysis (RDA) developed by Lisitsyn *et al* [6], has been suggested as a promising approach for DNA cloning. RDA is a method for isolating DNA fragments that are present in two nearly identical complex genomes. RDA is a subtractive hybridization method which is different from conventional methods [7-9] in that it uses representation of the genomes that have a reduction in complexity. Representation is generated by a polymerase chain reaction (PCR)-based size selection which applies the restriction fragments of both genomes. RDA takes advantage of both subtractive hybridization and DNA reassociation kinetics to favor the reiterated PCR amplification of the difference between the two genomes. In this study, we used *M. arthritidis* DNA fragments as tester sequences and *M. pulmonis* DNA fragments as driver sequences in the hybridization reaction to process RDA. The process of identifying a *M. arthritidis* specific DNA fragment from RDA products is described in this report.

Corresponding author: Dr. Jwo-Farn Chiou, Graduate Institute of Microbiology, College of Medicine, National Taiwan University, No.1, Sec. 1, Jen-Ai Road, Taipei, Taiwan, ROC.

Materials and Methods

Microorganisms

M. arthritidis ATCC 19611 and a clinically isolated strain of *M. pulmonis* were kindly provided by Dr. Weng Chang-Nan. Some strains of *M. arthritidis* were clinical isolates provided by the Laboratory Animal Center of the College of Medicine of National Taiwan University. The *M. pneumoniae* strain FH was provided by Professor Huang Mei-Hui. Other organisms except for mycoplasmas were clinical isolates provided by the Laboratory Animal Center of the College of Medicine of National Taiwan University and by National Taiwan University Hospital.

Culture conditions

Mycoplasmas were grown in BBL mycoplasma broth (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) supplemented with 10% swine serum. Other organisms were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, MI, USA).

Representational difference analysis

RDA was performed as that previously described by Lisitsyn *et al* [6]. The anchor adapters used in this study were the same sequences as that described by Lisitsyn *et al* [6]. The restriction enzyme *Hind*III was used for digesting genomic DNA samples from both *M. arthritidis* ATCC 19611 and *M. pulmonis* strains, and corresponding anchor adapters were used for subsequent PCR amplification to prepare amplicons.

After two rounds of competitive hybridization, the RDA products were again digested with the restriction enzyme to remove the adapters, and then the digested products were cloned into plasmid pUC19, which had been previously digested with the *Hind*III restriction enzyme and treated with calf intestinal alkaline phosphatase. The clones obtained were further analyzed to verify *M. arthritidis* specificity of the insert DNA fragment.

Southern blot analysis

Genomic DNA (2 µg) was digested with *Hind*III restriction enzyme and the purified product was run through 1% agarose gel electrophoresis and blotted onto a nylon filter (Hybond-N, Amersham). DNA probe was prepared by purifying the RDA clone insert (MA 194) or PCR product using MA194 as template (MA-PCR), and then labeling with digoxigenin by PCR according to the manufacturer's instructions. Hybridization was also performed according to the manufacturer's instructions.

Dot blot analysis

1 µL DNA (1 µg/µL) samples prepared from the bacterial genome were blotted onto a Hybond-N nylon filter. The filter was then analyzed by hybridization with digoxigenin-labeled MA194 .

Cloning and sequencing of different products

The different products were digested with *Hind*III and cloned into the *Hind*III site of plasmid pUC19. Double stranded plasmid DNA was prepared using a miniprep column (Qiagen), and sequenced with a sequenase kit (USP) according to the manufacturer's instructions. Resulting sequences were compared to the GenBank database with the FASTA program.

Results and Discussion

Products of *M. arthritidis* from RDA

As shown in figure 1, the RDA products with sizes ranging from 154 bp to 517 bp were obtained after performance of the second cycle. The individual bands

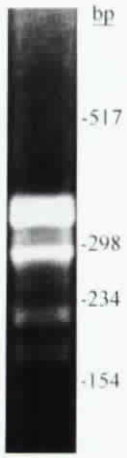

Fig. 1. The products of the second round of RDA. The sizes of the molecular markers are indicated on the right.

of RDA products were excised from agarose and digoxigenin-dUTP labeled by PCR with corresponding primers. After removal of the adapters, the digoxigenin-labeled PCR products were used as probe in dot blot hybridization with both *M. arthritidis* and *M. pulmonis* genomic DNAs. The RDA product of the 294 bp fragment was hybridized with *M. arthritidis* DNA only, whereas the other fragments were hybridized with the DNAs from both *M. arthritidis* and *M. pulmonis* (data not shown).

DNA sequencing and analysis of MA194

The nucleotide sequence of *M. arthritidis* in the 294 bp RDA product was determined by treating the product with *Hind*III restriction enzyme to remove the adapters at two ends of the product. The treated product was ligated with calf intestine alkaline phosphatase treated plasmid pUC19 and transformed into the *E. coli* DH5α strain. The inserted DNA obtained from the transformed *E. coli* was sequenced. As shown in figure 2, the inserted DNA contained 194 nucleotides. This DNA fragment was named as MA194. No open reading frame was found on comparing this fragment with DNA sequences in the Genbank database, however, the nucleotide sequence from 90 to 190 was shown to have 81% identity with the *sce* 659 fragment nucleotide sequence

AAGCTTGCATTGCTAGAGGTGGATGTAGTTGTCGGTTCTTTTGCAACTAATT
TTCAATTTCTTCTAGTAGTTTTTTAGCATCTTCTTTAAGTTTATCTTCTCTA
TCTTGAAGCATTTTTAGTTTATCTTTAGCTTCTTTTAAGGTTTCTTTAGACA
GATCAATAGTTGTAGCAAGCTCTTCTGCTAAAAGCTT

Fig. 2. Nucleotide sequence of MA194. Primers used for preparation of digoxigenin labeled MA194 and the specificity test by PCR are underlined.

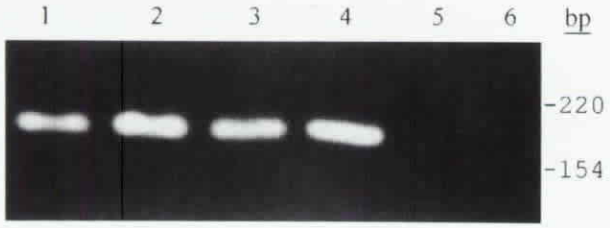

Fig. 3. Specificity test of *Mycoplasma arthritidis* primers designed from MA194 by PCR. 1. *M. arthritidis* 01; 2. *M. arthritidis* 07; 3. *M. arthritidis* ATTC 19611; 4. *M. arthritidis* 194; 5. *M. pulmonis*; and 6. *M. pneumoniae*.

of the *Saccharomyces cerevisiae* chromosome v lambda clone 6592 from 8730 to 8830 (data not shown).

Specificity test of MA194

To further verify the specificity of MA194, digoxigenin labeled MA194 was prepared by PCR with a pair of primers shown in figure 2. The digoxigenin labeled MA194 was then titrated with 1 μg of *M. arthritidis* DNA. A strong signal was found by using 50 ng digoxigenin labeled MA194 (data not shown). For the specificity test, 50 ng digoxigenin labeled MA194 was used to hybridize with genomic DNAs from various microorganisms including *Pseudomonas aeruginosa, Pseudomonas fluorescens, Acinetobacter baumannii, Burkholderia cepacia, Alcaligenes faecalis, Moraxella* species, *E. coli, Shigella sonnei, Salmonella* species, *Serratia marcescens, Citrobacter diversus, Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter cloacae, Morganella morganii, Providencia rettgeri, Aeromonas hydrophila, Vibrio* species, *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Stomatococcus bovis, Streptococcus pyogenes, Streptococcus faecalis, Streptococcus sanguis, Streptococcus agalactiae, M. pulmonis*, and *M. pneumoniae*. No signal was detected from the DNA spots of various microorganisms (data not shown). As shown in figure 3, all of the genomic DNAs from various strains of *M. arthritidis* were amplified by PCR while DNA from strains of *M. pulmonis* and *M. pneumoniae* were not amplified. As shown in figure 4, the minimal amount of genomic DNA which could be detected in PCR was 100 pg. For confirmation of the results of hybridization test, 1 μg genomic DNA from various microorganisms was used as a template during the PCR amplification. The RDA product was found only when DNA of *M. arthritidis* was used as template but not when DNA from other microorganisms was used as template (data not shown).

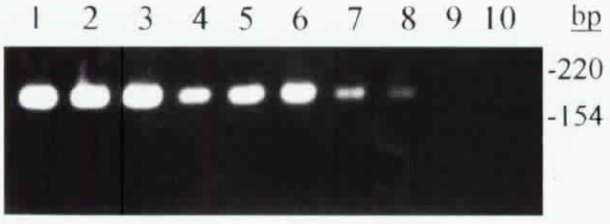

Fig. 4. Determination of the minimal concentration of *M. arthritidis* DNA required for PCR detection. The concentrations of DNA used for lanes 1 to 10 are as follows: 1. 500 ng; 2. 100 ng; 3. 50 ng; 4. 10 ng; 5. 5 ng; 6. 1.0 ng; 7. 0.5 ng; 8. 0.1 ng; 9. 0.05 ng; and 10. 0.01 ng.

RDA has been used in studies to identify DNA losses, amplifications in tumors [10-12], and DNA sequences from unknown pathogens in infected tissues [13]. RDA has also been used to identify binary polymorphism and polymorphism linked to a trait of interest [6,14,15]. In the present study, we obtained an *M. arthritidis* DNA fragment (MA194) by RDA. We verified that MA194 is *M. arthritidis* specific by hybridization and PCR amplification. These results indicate the ability of RDA to detect gene differences between species of bacteria regardless of whether they are in the same genus or group.

The finding of this study that DNA MA194 is a *M. arthritidis* specific fragment indicates that the use of labeled MA194 as a probe or the design of primers from MA194 as in this study for PCR detection might provide an alternative method for identifying species of mycoplasma from the isolates of the infected rodents. The use of the MA194 as probe and in the design of primers for PCR detection may therefore be of use in studies to determine the prevalence of *M. arthritidis* infection among colonies of rodents.

Acknowledgment

This article was supported in part by grants from the National Science Council (NSC 83-0409-B-002-204; 84-2321-B-002-133).

References

1. Kirchoff H. *Mycoplasma* infections of laboratory animals: current status. Isr J Med Sci 1987;23:775-7.
2. Minion FC, Brown MB, Cassell GH. Identification of cross-reactive antigens between *Mycoplasma pulmonis* and *Mycoplasma arthritidis*. Infect Immun 1984;43:115-21.
3. Cassell GH, Hill AC. Murine and other small-animal Mycoplasmas. In: Barile MF, Razin S, eds. The Mycoplasmas. Vol. 2. New York: Academic Press 1979:235-73.
4. Ferebee A, Simoneau P, Chang J, Barile MF, Hu PC. Differential detection of *Mycoplasma pulmonis* and *Mycoplasma arthritidis* with species-specific DNA probe. Diagn Microbiol Infect Dis 1992;15:411-15.
5. Matsuzaki M, Harasawa R, Kimizuka F, Koshimizu K. A non-

radioactive DNA probe for the detection of *Mycoplasma pulmonis* in murine mycoplasmosis. Microbiol Immunol 1989; 33:129-33.
6. Lisitsyn NA, Lisitsyn NM, Wigier M. Cloning the differences between two complex genomes. Science 1993;259:946-51.
7. Kunkel LM, Monaco AP, Middlesworth W, Ochs HD, Latt SA. Specific cloning of DNA fragments absent from the DNA of a male patient with an X chromosome deletion. Proc Natl Acad Sci USA 1989;82:4778-82.
8. Wieland I, Bolger G, Asouline G, Wigler M. A method for difference cloning: gene amplification following subtractive hybridization. Proc Natl Acad Sci USA 1990;87:2720-4.
9. Wieland I, Bohm M, Bogatz S. Isolation of DNA sequences deleted in lung cancer by genomic difference cloning. Proc Natl Acad Sci USA 1992;89:9705-9.
10. Lisitsyn NA, Lisitsina NM, Dalbagni G, Barker P, Sanchez CA, Gnarra J, Linehan WM, Reid BJ, Wigler MH. Comparative genomic analysis of tumors: detection of DNA losses and amplification. Proc Natl Acad Sci USA 1995;92:151-5.
11. Schutte M, Costa LT, Hahn SA, Moskaluk C, Hoque ATMS, Rozenblum E, Weinstein CL, Bittner M, Meltzer PS, Trent JM, Yeo CJ, Hruban RH, Kern SE. Identification by representation difference analysis of a homozygous deletion in pancreatic carcinoma that lies within the BRCA2 region. Proc Natl Acad Sci USA 1995;92:5950-4.
12. Tsuchiya H, Tsuchiya Y, Kobayashi T, Kikuchi Y, Hino O. Isolation of genes differentially expressed between the Yoshida sarcoma and long-survival Yoshida sarcoma variants: origin of Yoshida sarcoma revisited. Jpn J Cancer Res 1994;85:1099-104.
13. Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS. Identification of Herpes virus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 1994; 266:1865-9.
14. Lisitsyn NA, Segre JA, Kusumi K, Lisitsyn NM, Nadeau JH, Frankel WH, Lander ES. Direct isolation of polymorphic markers linked to a trait by genetically directed representation difference analysis. Natl Genet 1994;6:57-63.
15. Toyota M, Canzian F, Ushijima T, Hosoya Y, Kuramoto T, Serikawa T, Ima K, Sugimura T, Nagao M. A rat genetic map constructed by representational difference analysis markers with suitability for large-scale typing. Proc Natl Acad Sci USA 1996; 93:3914-9.