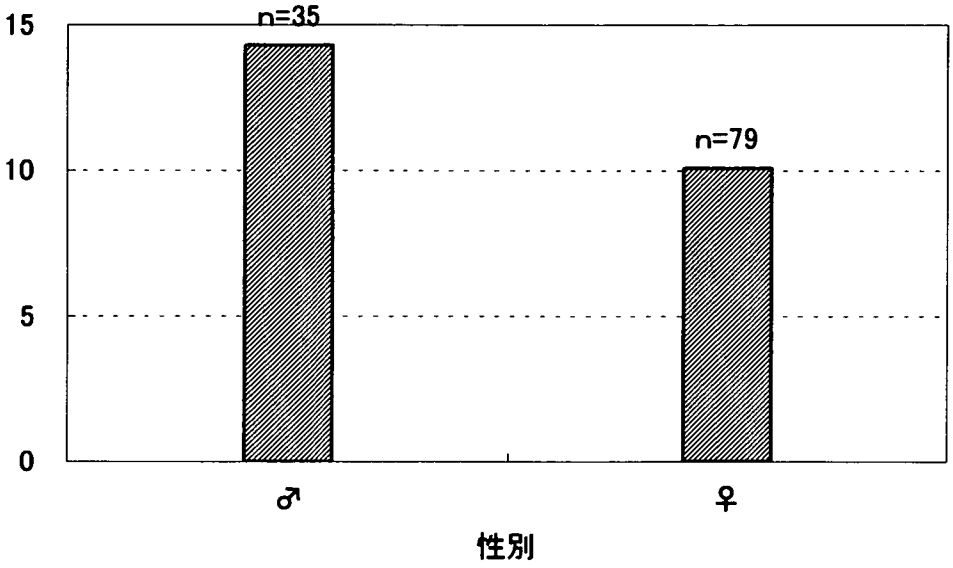

図1. 抗HEV 抗体保有状況の性差

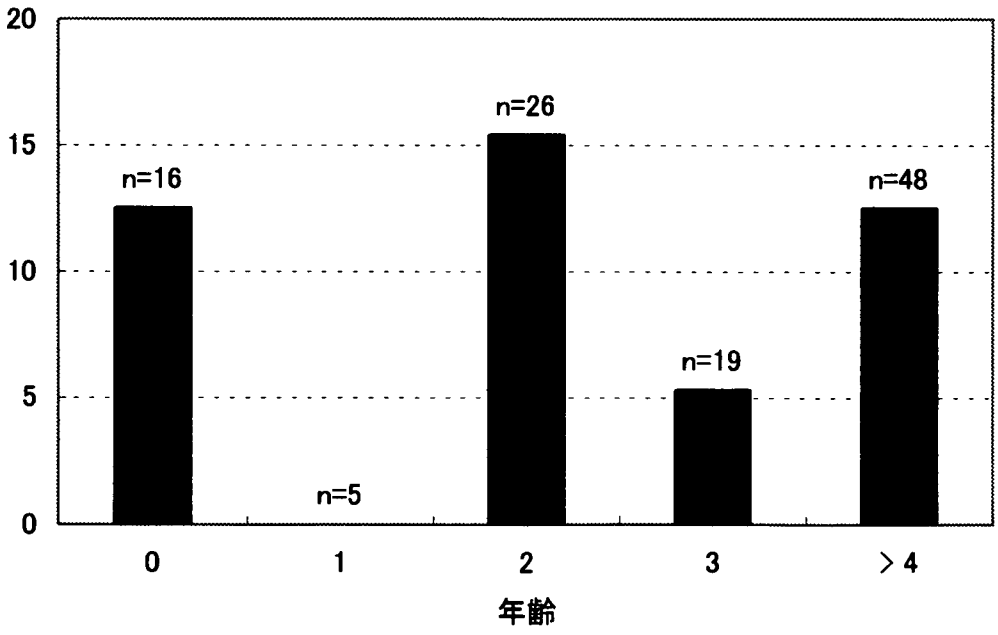

図2. 抗HEV 抗体陽性個体の年齢分布

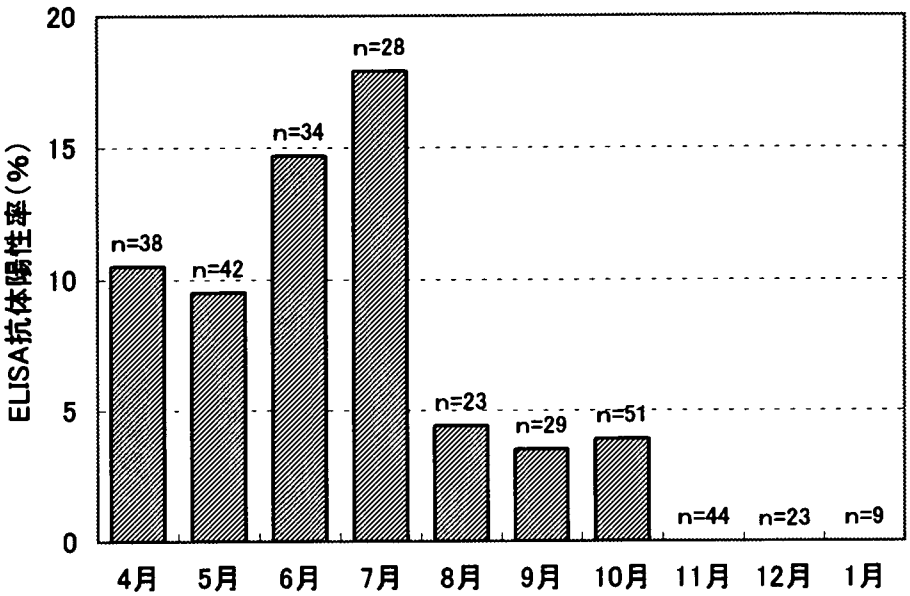

図 3. 抗 HEV 抗体陽性率の季節変動

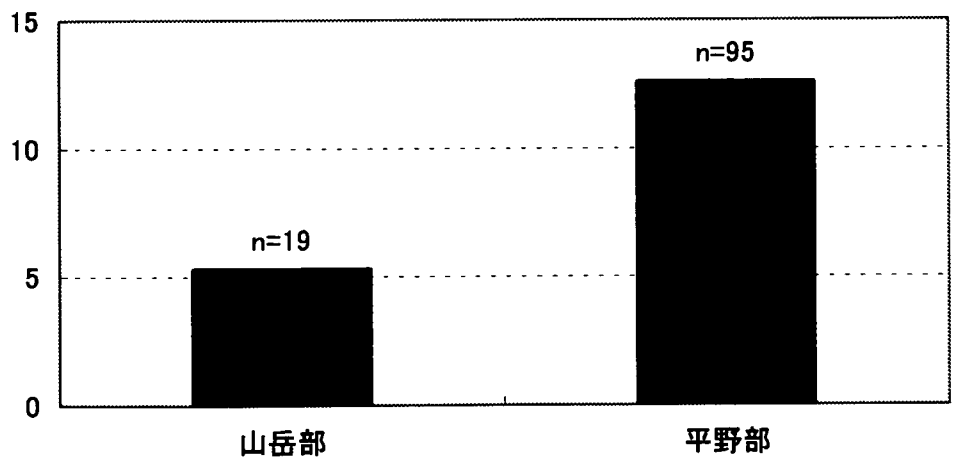

図4. 抗HEV抗体陽性個体の分布

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表雑誌名	巻	ページ	出版年
Uda A, Tanabayashi K, Fujita O, Hotta A, Yamamoto Y, Yamada A.	Comparison of whole genome amplification methods for detecting pathogenic bacterial genomic DNA using microarray.	Jpn J Infect Dis.	60	355-361	2007
Yamana H, Ito H, Ito T, Murase T, Motoike K, Wakabayashi K, Otsuki K.	Strong antiviral activity of heated and hydrated dolomite--preliminary investigation.	*J. Vet. Med. Sci.*	69	217-219	2007
Guo CT, Takahashi N, Yagi H, Kato K, Takahashi T, Yi SQ, Chen Y, Ito T, Otsuki K, Kida H, Kawaoka Y, Hidari KI, Miyamoto D, Suzuki T, Suzuki Y.	The quail and chicken intestine have sialyl-galactose sugar chains responsible for the binding of influenza A viruses to human type receptors.	Glycobiology	17	713-724	2007
Shinya K, Watanabe S, Ito T, Kasai N, Kawaoka Y.	Adaptation of an H7N7 equine influenza A virus in mice.	J. Gen. Virol.	88	547-553	2007
伊藤壽啓	鳥インフルエンザとパンデミック	炎症と免疫	15	80-85	2007
伊藤壽啓	高病原性鳥インフルエンザ	生物の科学遺伝（別冊）	20	172-175	2007
伊藤壽啓	野鳥と鳥インフルエンザとの関わり	鶏病研究会報	43	9-14	2007

伊藤壽啓	連載《インフルエンザ講座》宮崎県及び岡山県の養鶏場で発生した高病原性鳥インフルエンザの感染ルート究明	インフルエンザ	9	9-10	2007
伊藤壽啓	10 年前の鳥インフルエンザ・イン・ホンコン	インフルエンザ	9	64-67	2007
Borghan, M. A., Mori, Y., El-Mahmoudy, A-B., Ito, N., Sugiyama, M., Takewaki, T. & Minamoto, N.	Induction of Nitric Oxide Synthase by Rotavirus Enterotoxin NSP4: Implication for Rotavirus Pathogenicity.	J. Gen. Virol.	88	2064-2072	2007
Okazaki, K	Proteolytic cleavage of glycoprotein B is dispensable for in vitro replication but required for syncytium formation of pseudorabies virus.	J. Gen. Virol.	88	1859-1865	2007

Jpn. J. Infect. Dis., 60, 355-361, 2007

Original Article

Comparison of Whole Genome Amplification Methods for Detecting Pathogenic Bacterial Genomic DNA Using Microarray

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(Received January 23, 2007. Accepted August 17, 2007)

SUMMARY: The genetic diagnosis of pathogenic agents using microarrays has the advantage of high-throughput detection, but a relatively large amount of DNA sample is required. To obtain a sufficient amount of DNA for molecular diagnoses, several whole genome amplification (WGA) methods have been proposed. In this study, using *Francisella tularensis* and *Escherichia coli* as models, we compared four WGA methods in terms of their efficiency of amplification of whole genomic DNA in order to identify the most suitable method for preparing DNA to be used for microarray analysis. It was possible to obtain more than 1.5 μg of products from 10 ng of *F. tularensis* and *E. coli* genomic DNA using four methods, but biases in the amplification of bacterial genes were least prominent in the multiple displacement amplification (MDA) or OmniPlex WGA. When the amplified DNAs were applied to microarray slides consisting of 32 different genes probes, DNAs amplified by Phi29 v2 of MDA and OmniPlex WGA showed high signal intensity as well as a high signal-to-noise ratio for all 32 genes. These results indicate that Phi29 v2 and OmniPlex WGA are useful methods for obtaining sufficient DNA from a limited amount of samples for the detection of microbes using microarrays.

INTRODUCTION

Early diagnosis is the prerequisite for giving not only appropriate but also prompt antibiotic treatment to patients with microbial infections. Conventional laboratory diagnoses of pathogenic agents are commonly conducted by cultivation and serological tests such as enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay (IFA), agglutination, and Western blotting. However, serological tests cannot be used until specific antibodies appear in the patient's serum. Genetic diagnosis using a polymerase chain reaction (PCR) technique is advantageous because the genetic material can be rapidly detected with high sensitivity and specificity from a small sample of nucleic acid. The microarray is a high-throughput bioinformatic tool for biological gene expression profiling as well as for the simultaneous detection of various pathogenic agents (1-3). However, 1-10 μg of fluorescently labeled DNA is required for microarray analysis. Therefore, techniques to amplify the sample DNA are required only if a limited amount of DNA template is available.

Degenerate oligonucleotide (DOP)-PCR (4-7) has been reported to be useful in amplifying small amounts of DNA. The 6MW primer, designed for DOP-PCR by Telenius et al. (7), has six-nucleotide random sequences flanked by 5′ and 3′ anchor sequences. It is thought that the primer can anneal to any DNA sequence in the whole genome region. This product is often used in comparative genomic hybridization (CGH) arrays (4-6). Multiple displacement amplification (MDA) using phi29 DNA polymerase (8-13) involves strand displacement DNA synthesis on single- and double-strand DNA templates by random hexamer annealing at multiple sites. This enzyme has the ability to synthesize DNA fragments greater than 70 kb (14) and has 3′ → 5′ exonuclease activity resulting in a low error rate (15). The products obtained using MDA are also used for CGH analysis (16) as well as plasmid DNA sequencing without cloning (10), and single-nucleotide polymorphism (SNP) genotyping (17). OmniPlex library technology (18-21) is a novel method for performing whole genome amplification (WGA). In OmniPlex WGA, the genomic DNA randomly fragmented (mean predictable fragment size ~0.4 kb) is ligated to universal priming sites and then amplified with universal primer by PCR. The products of OmniPlex WGA are used for genotyping (18) and CGH (20, 21).

Francisella tularensis, which causes tularemia, is a Gram-negative facultative intracellular bacterium which is listed as a category A bioterrorism agent by the Centers for Disease Control and Prevention (CDC). Humans are infected with *F. tularensis* by the bite of arthropod vectors, contact with infected animals, and inhalation of contaminated dust (22-24). Tularemia can be treated by early application of proper antibiotics, but diagnosis from the clinical symptoms of tularemia is usually difficult. Therefore, it is important to develop a fast diagnostic system such as detection of the causative agent, *F. tularensis*.

In this study, we compared representative WGA methods to find out which would be the most suitable for the detection of DNA from pathogenic bacterial agents such as *F. tularensis* with a 60-mer oligo-DNA microarray.

MATERIALS AND METHODS

Bacterial strains and DNA extraction: *F. tularensis* subsp. *tularensis* strain Schu was grown in Eugon agar plates supplemented with 8% chocolatized sheep blood, and genomic DNA was extracted using SepaGene (Sanko Pharmaceutical Co., Tokyo, Japan), as described previously (25). *Escherichia coli* K12 ER2925, obtained from New England BioLabs (NEB, Beverly, Mass., USA), was cultured in Luria-Bertani (LB)

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medium at 37°C overnight and the genomic DNA was then extracted with a DNA Isolation Kit for Cells and Tissue (Roche, Mannheim, Germany).

Amplification by DOP-PCR: The 6MW primer (5′-CCG ACTCGAGNNNNNNATGTGG-3′), designed by Telenius et al. (7), was synthesized by Hokkaido System Science (Hokkaido, Japan). DOP-PCR was performed on a GeneAmp PCR System 9700 (Perkin Elmer, Foster City, Calif., USA) in a 20-μl reaction mixture consisting of 1 unit of Takara Ex Taq (Takara, Shiga, Japan), 1 × PCR buffer supplied with the enzyme, 2 μM of primer, 250 μM of each dNTP, and 10 ng of genomic DNA. The reaction conditions were as follows: initial denaturation at 95°C for 5 min, 5 cycles of 95°C for 0.5 min, 30°C for 0.5 min, ramp at 0.7°C/s to 72°C, 72°C for 1.5 min followed by 35 cycles of 95°C for 0.5 min, 62°C for 0.5 min, 72°C for 1.5 min, and final extension at 72°C for 7 min. The amplified DNAs were stored at −30°C until use.

MDA: Bacterial genomic DNA was amplified using the GenomiPhi DNA Amplification Kit (GE Healthcare, Piscataway, N.J., USA) and the GenomiPhi DNA Amplification Kit version 2 (GE Healthcare), according to the manual supplied by the manufacturer. In this study, these two kits were designated Phi29 v1 and Phi29 v2, respectively. Briefly, 10 ng of genomic DNA was heat-denatured at 95°C for 5 min and then chilled on ice for 5 min. After the addition of enzyme solution, each sample was incubated at 30°C for 18 h (Phi29 v1) or for 2 h (Phi29 v2). The DNA polymerases were heat-inactivated at 65°C for 10 min, and the samples were stored at −30°C until use.

OmniPlex WGA: The DNA samples were amplified using the GenomePlex Whole Genome Amplification Kit (Sigma, Poole, UK) according to the manufacturer's manual. Briefly, 10 ng of bacterial genomic DNA was treated with Fragmentation Buffer at 95°C for 4 min and chilled on ice. Library Preparation Buffer and Library Stabilization Buffer were added to the samples. The samples were incubated at 95°C for 2 min and chilled on ice. After the addition of Library Preparation Enzyme, the DNAs were sequentially incubated at 16°C for 20 min, 24°C for 20 min, 37°C for 20 min, and 75°C for 5 min. The DNAs were amplified with Amplification Master Mix containing 12.5 units of Takara ExTaq (Takara) under the following conditions: initial denaturation at 95°C for 3 min, 14 cycles of 95°C for 15 s and 65°C for 5 min. The samples were stored at −30°C until use.

Real-time PCR: The primers and probes targeting three regions of the *F. tularensis* or *E. coli* genome sequences were designed using Primer Express 3 software (Applied Biosystems, Foster City, Calif., USA) and synthesized by Hokkaido System Science (Table 1). Real-time PCR was performed with an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems) with the aid of SDS software v1.3. Twenty microlitres of reaction mixture consisting of

Table 1. Primers and Taqman probes for real-time PCR used in this study

Name	Position	Length	Tm	Final conc. (μM)	Sequence
Francisella tularensis subsp. *tularensis* schu 4 (NC_006570)					
ATP synthase subunit A: *atpA* (FTT0062)/amplicon size: 129bp					
Ft/atpA-F	63123	25	58	0.6	5'-TTGACTAAACAAGCATGGGCTTATA-3'
Ft/atpA-P	63171	27	69	0.6	5'-FAM-AGACCGCCTGGACGTGAAGCTTATCCT-Tamra-3'
Ft/atpA-R	63251	24	59	0.1	5'-GTTGACTCTTGCAGCTCTTTCAAG-3'
succinate dehydrogenase, catalytic and NAD/flavoprotein subunit: *sdhA* (FT0074)/amplicon size: 76bp					
Ft/sdhA-F	75023	24	58	0.8	5'-GGGATGTCAAGGAACTATGATCCT-3'
Ft/sdhA-P	75048	29	69	0.8	5'-FAM-CTAATCAAGCAGAAAGAACTTGCGCAGCA-Tamra-3'
Ft/sdhA-R	75098	19	59	0.1	5'-GAGCATGCCCGGTCCTATC-3'
glyceraldehyde-3-phosphate dehydrogenase: *gapA* (FT1368c) / amplicon size: 108bp					
Ft/gapA-F	1414435	27	58	0.6	5'-TGCATATACAGGTGACCAAAATACTTT-3'
Ft/gapA-P	1414406	28	70	0.6	5'-FAM-ATGCTCCTCATGCGAAAAATGACTTCCG-Tamra-3'
Ft/gapA-R	1414328	24	59	0.1	5'-GCAGCACCAGTTGAGTTAGGTACA-3'
Escherichia coli K12 (NC_000913)					
putative outer membrane protein: *ycdS* (b1024)/amplicon size: 75bp					
Ec/ycdS-F	1090789	20	59	0.6	5'-CCTACGCGCAGTGAAAGTGA-3'
Ec/ycdS-P	1090759	19	69	0.6	5'-FAM-ATTGCCGATCGCGCCCTCG-Tamra-3'
Ec/ycdS-R	1090715	25	60	0.1	5'-CAGAATTTCTAATGCAGCGTATTGG-3'
putative fimbrial-like protein: *ybgD* (b0719)/amplicon size: 69bp					
Ec/ybgD-F	751827	21	59	0.6	5'-CCGGGAGCATCATAGCAATAA-3'
Ec/ybgD-P	751804	23	69	0.6	5'-FAM-TGGCCGTCGACATTCGCTTGATC-Tamra-3'
Ec/ybgD-R	751759	22	59	0.1	5'-TCAGAAGCAGGCAGATCACAGT-3'
glyceraldehyde-3-phosphate dehydrogenase: *gapA* (b1779)/amplicon size: 78bp					
Ec/gapA-F	1860814	20	59	0.6	5'-TCAACGGTTTTGGCCGTATC-3'
Ec/gapA-P	1860836	24	69	0.6	5'-FAM-TCGCATTGTTTTCCGTGCTGCTCA-Tamra-3'
Ec/gapA-R	1860891	22	60	0.1	5'-TGATTGCAACGATCTCGATGTC-3'

-F, Forward primer; -R, Reverse primer; -P, Taqman probe. Probes were labeled at 5' end with 6-carboxy-fluorescein (FAM) and 3' quencher, Tamra. These primers and probes were designed using Primer Express software.

1×Premix ExTaq (Takara), the passive reference dye (Rox), primers 600 or 800 nM, probe 100 nM, and diluted sample DNA were used. The PCR conditions were as follows: initial denaturation at 95°C for 10 s, 40 cycles of 95°C for 10 s and 60°C for 35 s. The biases of amplification were assessed by relative quantification using the $2^{-\Delta\Delta Ct}$ method. The WGA reactions diluted 100-fold in TE buffer were subjected to real-time PCR along with 20 ng of bacterial genomic DNA. Data on relative quantifications were analyzed using auto-threshold (Ct) and auto-baseline settings.

Microarray design, construction, and preparation: Synthetic oligonucleotide DNAs spotted onto microarray slides were designed using Array Designer 3.01 (Premier Biosoft International, Palo Alto, Calif., USA) with the default setting parameters. The search nucleotide length was 60 mer, Tm was 75°C ± 5.0°C, cross-homology was avoided, and the sense probe was designed. Nucleotides able to form hairpin loops (<–3.0 kcal/mol) and self-dimers (<–3.0 kcal/mol) were excluded. These nucleotide sequences are shown in Table 2. The probes for *F. tularensis* were designed so that they could

Table 2. Oligo-DNA used for microarray

Code	Gene name	Accession number	Position	Sequence (60mer)**	Tm
Francisella tularensis subsp. *tularensis* Schu 4, complete genome (NC_006570)					
F01	*atp*A	FTT0062	63234	AGAGCTGCAAGAGTCAACGAAGAATATGTCGAGAAATTTACAAATGGTGAAGTGAAAGGT	73.7
F02	*sdh*A	FTT0074	75142	TTTCTATACGGAGTGGTTTGCTGTTGATTTGGTTAAGGCGGATGATGGTAGTATTGCTGG	75.3
F03	*val*A	FTT0109	115455	AGTCTCAGGGATCTAGTAATCTAAGTGGAGAAATGACAAACCATCAGAAAGTAGGCACTC	73.4
F04	*val*B	FTT0110	117538	TTTGAAGTAACAAGTGGTACTCTAGCAACTCAATGTGGCGATGAGCCTGCGATGTTATTT	75.3
F05	*par*C	FTT0396	397224	AGGTTTCAAGTGCTGCGGTAATGGAGCAAATCGCTAATGAACTTAAGCAACAGAAGATTA	74.8
F06	*asd*	FTT0425c	439364	AGGGTCTAGAACTAGAGTACTATCTTTGTCTAAGCGTAGTGTCGATGCAGCGTCTATCCA	75.0
F07	*aro*A	FTT0588	608258	CCTTTGACAATAATAGCTAAATCTTTGGATGGTGGTTATATAGAAGTTGATGGCGAGAAG	71.3
F08	*mdh*	FTT0535c	556996	AGCAACAATTTCGCCACCACCACTTCTAGTTCTCGATACTATAGCATCTAAACGCTCTTG	75.0
F09	*alr*	FTT0573	591250	CTATGGCTGGTAGAATGAGTATGGATGGTCTGACAGTATCGTTAGGAATTAATGAATACG	72.1
F10	*fop*A	FTT0583	599581	GGAACTCCTAACTCTCCATCAGGTGCTGGTGCTAACTTCACAATCGGTTATAACATCAAT	74.9
F11	*gal*E	FTT0791	809639	CTCATGCCTTATGTCGCGCAAGTAGGTGCTGGTAAACTAGCTAAACTTAGTATCTTTGGT	75.0
F12	*mgl*B	FTT1276	1297740	GCGAATTTGGTTATGGAATTAATATCAATGAAGGCGAAGATGATGAAACTGCTAATCCTA	71.6
F13	*igl*D	FTT1356c	1398469	TTATAAGGATAAGACCTGTCTGCAAACTTTCAACATCTAAATCAAAGCTAACGATTCCCT	71.8
F14	*igl*C	FTT1357c	1399233	CAGTAGGATCAGTTCTCACATGAATGGTCTCGCCACTTGTTACCTGTTGTCTTGTTATCA	74.8
F15	*pdp*D	FTT1360c	1401540	CCTCTAGCTGGTACAGTTGCTAAGACACTAAAACCTTGTGAACTTCTAATCTCTTCATGT	73.0
F16	*min*D	FTT1606	1672559	TAGTAACTTCTGGTAAAGGTGGTGTTGGTAAAACTACTTCAAGTGCCGCTGTTGCATATG	74.6
Escherichia coli K12, complete genoma (NC_000913)					
E01	*ykg*K	b0294	309995	GAGTACAGCTTGGCCTCAGCATTACGCCGATGGGTATACACTGTCTTCACACTACAATTT	76.6
E02	*eae*H	b0297	313979	TTCGCTGGAAATGCTTTATCCGATTTATGATACGCCGACAAATATGTTGTTCACTCAGGG	74.8
E03	*ykg*A	b0300	316254	CGTCTGCTGTAGCCAGATTTCTGTGCGATATCTTCGATTGAAATAGGGTGCTCAAGATTG	75.4
E04	*bef*T	b0314	329505	GTTCGTATTGTTTATGGGCGACACTTCGTTCCTGCTTAATGCACTGGTGCTGAATGTTGG	77.0
E05	*yah*E	b0319	335371	GGTATTACGGTTGGTCAACATCTTCATATAGAGATGAGTCGTTGTCGGCGTTGGCTGTCC	76.6
E06	*yah*K	b0325	342108	ATGAAGATCAAAGCTGTTGGTGCATATTCCGCTAAACAACCACTTGAACCGATGGATATC	74.9
E07	*ybb*V	b0510	538174	GGTAAGTTTATTCACTTTATGGAACCGTTATCGCGTGTTTCATGGTTTGTCGGCGTCATC	75.3
E08	*ybb*Y	b0513	540812	AGTATCCCTTTACCCGTGAGTAGTGCGGTCATGCTGGTTTCTTATCTGCCTTTACTCTTT	76.1
E09	*ylb*A	b0515	542502	GGTTACAATCTTTCGAGTAAATATAGCTGAACGCTTCACCACGCCCTACACCATAACCAG	75.2
E10	*ybc*K	b0544	568525	CCATACTTATAGCACAAAGGGCAAATGAAGAAAGCGAGATAAAGTCAAGTCGGGTTAAAT	73.1
E11	*omp*T	b0565	584327	CTGAATCCCTCCTCAGAACTGTAGATATAGGAACCACCTCTGGCTGTAAAGCTATAACGG	74.9
E12	*ybg*D	b0719	751801	GCCACTTTATTGCTATGATGCTCCCGGTTTATATGGGTTGTCGTGACTTGTCCAAGATCT	76.0
E13	*ycd*Q	b1022	1085980	TATGTGTCGCAGCGATATGTGTCAATTCAATATTTAACGGTACACCGGCAAGTTGGACTG	75.6
E14	*ycd*R	b1023	1088229	CAATTGCGACGCATTTGCCAAACCTGATTCAAGGGTGAAGAACATGTCATAACCGAGTTT	76.4
E15	*ycd*S	b1024	1090071	GTTAATGCTGTCCAGGCTTGTTCAACCTCCAGATTAATATTACGTGGCTCGATCACTTCT	75.1
E16	*yci*Q	b1268	1323473	CGAATATTTTCAGCCATCTTCTTTTACCCTCCACGTCATCGTTGTTAATTTGGTTTCCGT	74.3
Position marker/*Homo sapiens* aryl hydrocarbon receptor (NM_001621)					
P	AHR	NM_001621	700	AGAGACCGACTTAATACAGAGTTGGACCGT	62.0

** The nucleotide length of AHR used as position control was 30mer.
The spotting layout of these nucleotides is show in Fig. 2A.

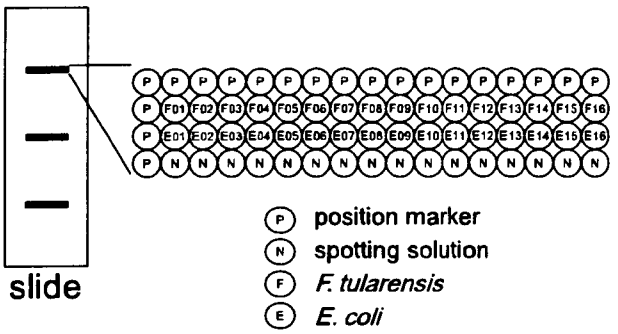

Fig. 1. Layout of the microarray. A mixture of AHR nucleotide conjugated with Cy3 and Cy5 (see Table 2) was used as position control (P). As negative control, spotting solution without nucleotides was used (N). The oligonucleotides representing 16 different genes (Table 2) of *F. tularensis* (F) and *E. coli* (E) genes were spotted in the second and third lanes, respectively. The first lane and the first rows were spotted with position markers (P).

specifically hybridize to amplicons from *F. tularensis* but not to those from *Francisella philomiragia*.

These 5′ amino-labeled nucleotides were synthesized and spotted onto GeneSlide (Toyokohan, Co., Tokyo, Japan) by the Hokkaido System Science. Briefly, 1 nl of nucleotide solution was spotted at a concentration of 100 μM. A mixture of AHR nucleotide conjugated Cy5 and Cy3 was used as the position control and spotted at a concentration of 10 μM. The slides were heat-fixed at 80°C for 1 h, followed by washing with 2 × SSC containing 0.5% SDS for 10 min, boiling with 2 × SSC containing 0.5% SDS for 10 min, rinsing with distilled water (DW) three times, and then drying by centrifugation.

The layout of the microarray is shown in Figure 1. The position of the subarrays prepared on a slide (1 × 3 inches) is schematically shown. Within each subarray, 60-mer oligonucleotide probes were spotted in a 17 × 4 configuration. There were 204 spots in total on each slide.

Hybridization, scanning, and data acquisition: Before fluorescent labeling, the DNAs amplified by the DOP-PCR, Phi29 v1, and Phi29 v2 methods as well as the genomic DNA extracted from *E. coli* were treated with 10 units *Rsa*I (NEB) and 10 units *Alu*I (NEB) at 37°C for 1 h. In contrast, the OmniPlex WGA samples were not treated with restriction enzymes because the average product size was predicted to be 400 base pairs (bp). After phenol/chloroform extraction and ethanol precipitation, the DNAs were dissolved in 5 mM Tris-HCl (pH 8.0) containing 1 mM EDTA buffer. Two to ten micrograms of DNA from *F. tularensis* or *E. coli* was denatured at 95°C for 5 min and labeled using the Ulysis Alexa Fluor 647 Nucleic Acid Labeling Kit (Molecular Probes, Inc., Eugene, Oreg., USA) or Ulysis Alexa Fluor 546 Nucleic Acid Labeling Kit (Molecular Probes) at 80°C for 15 min, respectively. The labeled DNAs were ethanol precipitated, washed twice with 70% ethanol, dried, and then dissolved in DW. After ethanol precipitation, the concentrations of labeled DNAs were measured by Nano Drop ND-1000 (Nano Drop Technologies, Rockland, Del., USA). The mixture of equal amounts of the *F. tularensis* and *E. coli* labeled DNA was filled up to 50 μl. The DNAs were denatured at 95°C for 3 min and mixed with 450 μl of hybridization buffer. The final mixture contained 4 μg/ml of each labeled DNA, 6× SSC, 5 × Denhardt's solution, 50 mM sodium phosphate, 0.5% SDS, and 20% formamide. The hybridization mixture (450 μl) was loaded into the well of a gasket slide (Agilent) and then the microarray slide was placed on the gasket slide. The DNA hybridization was conducted in a Hybridization Oven (Agilent) at 50°C overnight. The slides were then washed with 2 × SSC containing 0.5% SDS at 50°C for 5 min, washed twice with 1 × SSC at 50°C for 5 min, and then rinsed with DW at room temperature for 10 s. The microarray slides were dried with a SpinDryer Mini model 2350 (Wakenyaku, Kyoto, Japan) and then scanned under a green laser (546 nm; Alexa 546 and Cy3) and a red laser (633 nm; for Alexa 647and Cy5) at 100% laser power and 80% PMT at 10-μm resolution using ScanArray Express (Perkin Elmer). The images were analyzed by ScanArray Express software (Perkin Elmer), and the raw data were exported to a Microsoft Excel spreadsheet for further analysis.

RESULTS

Evaluation of amplification efficiency and biases of different WGA: We determined which WGA methods were useful to amplify DNA for the microarray assay to detect the genomic DNA of *F. tularensis* and *E. coli*. Ten nanograms of genomic DNAs extracted from *F. tularensis* strain Schu and *E. coli* K12 were amplified by the DOP-PCR, Phi29 v1, Phi29 v2, and OmniPlex WGA methods. After purification by NucleoSpin Extract II (Machery-Nagel, Düren, Germany), the amounts of amplified DNAs were measured (Fig. 2A). When 10 ng of genomic DNA extracted from *F. tularensis* was used as the template DNA, the DNA yields were 3.3, 2.6, 1.5, and 1.5 μg by DOP-PCR, Phi29 v1, Phi29 v2, and OmniPlex WGA, respectively. The yields of *E. coli* DNA obtained by these four methods ranged from 2.4 to 5.1 μg, which were greater than the yields of *F. tularensis* DNA. Using the Phi29 v1 method, large amounts of DNA were obtained even without the use of template DNA. These high yields might have resulted from the polymerization of random hexamer primers in the reaction mixture.

The amplified samples were subjected to 0.7% agarose gel electrophoresis to determine the amplicon size (Fig. 2B). The amplicons obtained with DOP-PCR appeared as smears from 0.3 to 3 kbp, with many ladder bands. The MDA products obtained with Phi29 v1 and Phi29 v2 were also found as smears (>0.5 kbp), but more evenly distributed. In the case of Phi29 v1, similar smear DNAs were produced without template DNA (Fig. 2B, Phi29 v1, lane 3). On the other hand, OmniPlex WGA produced DNA smears evenly distributed from 0.3 to 1 kbp.

The biases associated with each WGA method were assessed by relative quantification using the $2^{-\Delta\Delta Ct}$ method with real-time PCR. The target genes and sequences of primer pairs and Taqman probes are shown in Table 1. The amplification efficiency for each primer pair and Taqman probe set were 97 - 100% in all target regions (data not shown), so that we could analyze the biases of each WGA method by relative quantification. Relative quantities of DNA corresponding to the genes amplified by four different WGA methods were expressed as $2^{-\Delta\Delta Ct}$ in Fig. 2C. When the *sdh*A and *atp*A genes of *F. tularensis* were quantified by real-time PCR, the $2^{-\Delta\Delta Ct}$ value was 1.0, indicating that the genomic DNAs contained equal copy numbers of these genes. The DOP-PCR produced higher quantities of amplified DNA, suggesting that this method was prone to produce higher biases. Amplification efficiency was obviously different between two genes of *E. coli* when DOP-PCR was examined. MDA with both Phi29 v1 and Phi29 v2 appeared to produce the smallest biases

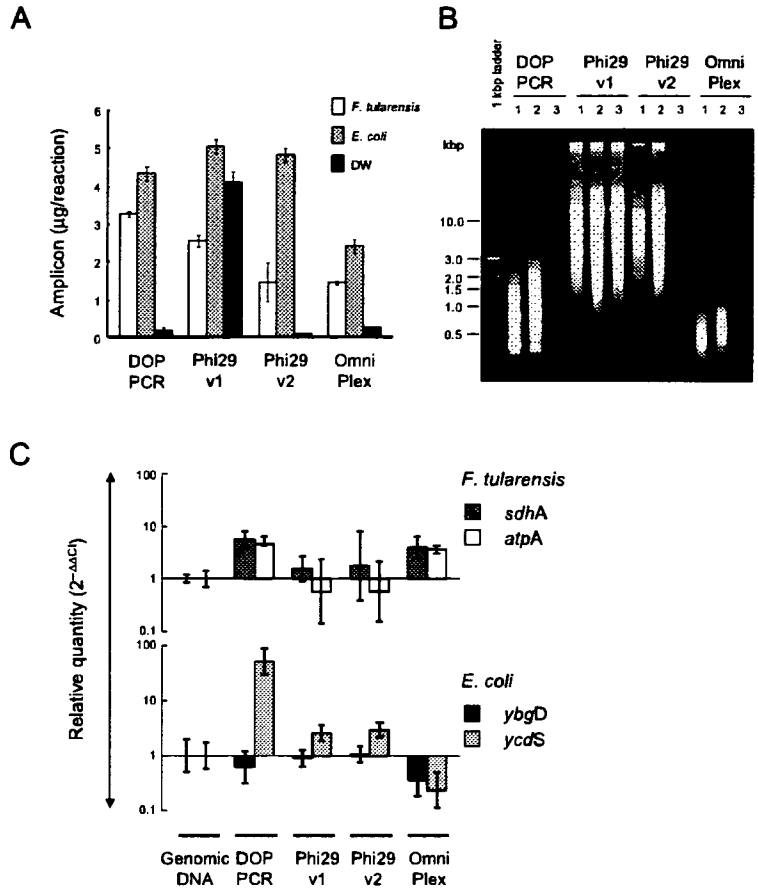

Fig. 2. Characterization of amplified DNAs by WGA methods. Bacterial genomic DNA was amplified by DOP-PCR, Phi29 v1, Phi29 V2, and OmniPlex WGA. All amplifications were done in triplicate. (A) The amounts of amplified DNA (μg/reaction) were calculated as concentration (μg/μl) × volume (μl). The vertical axis indicates the amount of amplified DNA per reaction. The bar indicates the standard error (SE). (B) Ten microliters of purified DNA from *F. tularensis* Schu (lane 1) and *E. coli* K12 (lane 2) was subjected to electrophoresis on 0.7% agarose gel. Amplicons obtained in the absence of template DNA were also included (lane 3). (C) Bias analysis in WGA amplicons using the relative quantification method. Real-time PCR analysis was performed to determine the amount of target genes relative to the *gapA* gene for each WGA. All amplifications were done in triplicate. Relative quantification was conducted using the $2^{-\Delta\Delta Ct}$ method with Applied Biosystems 7500 Real-time PCR System using SDS software. The bar indicates the range.

among the WGA methods tested, indicating that genomic DNAs from either *F. tularensis* Schu or *E. coli* were most uniformly amplified using MDA as compared with the other two WGA methods.

The reactivity of WGA amplicons to microarray: The amplified DNAs produced by DOP-PCR and MDA from 10 ng of each bacterial DNA and *E. coli* genomic DNA were treated with two restriction enzymes. The resulting DNA product appeared as a smear from 0.1 to 0.8 kbp (data not shown). Amplicons obtained by each method were labeled with Alexa 647 for *F. tularensis* and Alexa 546 for *E. coli*. The DNAs were mixed and applied to microarray slides. The results of scanning each microarray slide using two colors are shown in Fig. 3A. When amplicons produced by Phi29 v2, OmniPlex WGA, and the genomic DNA of *E. coli* were hybridized to a microarray, signals specific for each of 16 selected genes of *F. tularensis* or *E. coli* were observed. The intensity and signal-to-noise ratio (SNR) achieved by Phi29 v2, OmniPlex WGA, and the genome DNA of *E. coli* amplicons were higher than those by amplicons from DOP-PCR (Fig. 3B). The Phi29 v1 amplicons also gave significant signals, except for some genes from *F. tularensis*. Several genes were undetectable when amplicons from DOP-PCR were applied. These results indicate that Phi29 v2 and OmniPlex WGA are useful methods for obtaining sufficient DNA from a limited amount of samples for the detection of microbes using microarray.

DISCUSSION

Microarray analysis to detect the genomes of infectious agents commonly requires a large amount of nucleic acid samples, and amplification of the sample DNA is generally necessary. Therefore, we attempted to find the most useful WGA method to obtain DNA from pathogenic agents for microarray analysis. Various DNA amplification strategies have been employed for the detection of pathogenic agents using microarrays. Blaskovic and Barak (2) reported that amplified bacterial 16S rDNA was useful for the oligochip-based detection of tick-borne bacteria. Multiplex PCR, for which it is difficult to design primers, provides an amplified DNA sample for the simultaneous detection of several genes of pathogenic agents using microarrays (1). It was possible to use amplicons obtained by a random PCR that was a modified sequence-independent DNA amplification (SIA) technique (26) to detect multiple viruses using microarrays (3). We preferred WGA for universal microarray detection over PCR

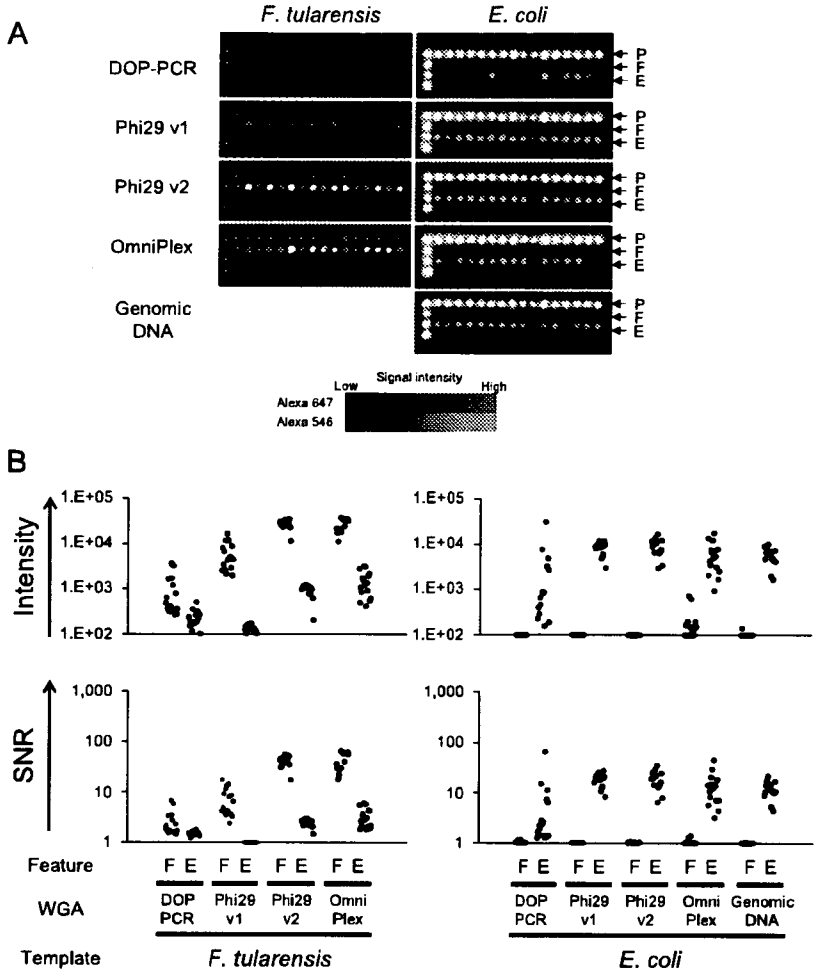

Fig. 3. Microarray analysis of DNA amplified by four WGA methods. (A) *F. tularensis* samples labeled with Alexa 647 (red image) and *E. coli* samples labeled with Alexa 546 (green image) were mixed and hybridized to microarray slides. After hybridization, microarray slides were 2-color scanned under a green laser (546 nm) and a red laser (633 nm) at 100% laser power and 80% PMT at 10-μm resolution using ScanArray Express (Perkin Elmer). (B) The image files (tif format) were analyzed by ScanArray Express software (Perkin Elmer). The raw data of the mean signal, the mean value of the local background, signal intensity (signal-background), and signal-to-noise ratio (SNR; signal/background) were exported to Microsoft Excel spreadsheet for analysis. As target genes were spotted in triplicate on a microarray slide as shown in Fig. 1, the average values of signal intensity and SNR for total 6 spots on two slides each gene are shown by dots.

for amplifying specific genes, since WGA seemed advantageous for detecting various pathogens simultaneously.

In this study, we compared four commercially available WGA methods–DOP-PCR, Phi29 v1, Phi29 v2, and OmniPlex WGA–with respect to amplification efficiency and bias (Fig. 2). OmniPlex WGA produced 1.5 μg and 2.4 μg products from 10 ng of *F. tularensis* and *E. coli* genomic DNAs, respectively. The amplicons obtained by the OmniPlex WGA method were efficiently hybridized with specific target spots on microarray (Fig. 3). Adequate amounts of products with low biases were also obtained by Phi29 v2 WGA, and the signal intensities and SNRs provided by the Phi29 v2 DNAs in the microarray assay were comparable to those achieved by DNAs obtained with OmniPlex WGA. These results indicate that Phi29 v2 and OmniPlex WGA are useful methods for obtaining sufficient DNA from a limited amount of samples, with no apparent biases, for the detection of microbes using microarrays.

DOP-PCR and Phi29 v1 did not appear suitable for the amplification of DNA applied for the microarray assay. Uneven amplification of genomic DNA by DOP-PCR was observed even when various conditions for PCR, including primer concentration and PCR programs, were examined (data not shown). A possible reason for the uneven amplification was that the potential annealing sites of six nucleotides at the 3′ end of the 6MW primer were less frequent in *E. coli* or *F. tularensis* than in humans (data not shown) (4,5). This bias in the DOP-PCR products may result in no induction of signals that in turn would lead to a false judgment concerning the presence of pathogens in the sample. A satisfactory amount of DNA with low bias was obtained by Phi29 v1, but signal intensity and SNR in the case of *F. tularensis* were not high enough for use in microarray analysis. Phi29 v1 produced large amounts of the DNAs even without DNA templates, which was probably due to the polymerization of random hexamers contained in the kit, as described previously (10,27).

All four WGA methods amplified genomic DNAs more efficiently from *E. coli* than from *F. tularensis* (Fig. 2). As the protocols used for the purification of bacterial genomic DNAs were different, amplification efficiency would be dependent upon the purity of these genomic DNAs. Alternatively, insufficient amplification using *F. tularensis* genomic

DNA as a template might have resulted from the difference in the GC contents of *F. tularensis* (32%) and *E. coli* (51%).

DOP-PCR and MDA are easy to perform but require restriction enzyme treatment for reduction of cross-hybridizations and to achieve high specificity. On the other hand, the procedure of OmniPlex WGA is slightly complicated but enzymatic treatment is unnecessary. Overall, both methods seem comparable in terms of the complexity of the procedures. The times required for the preparation of samples by DOP-PCR, Phi29 v2, and OmniPlex WGA were approximately 4 to 5 h, but it took 20 h to perform Phi29 v1.

In conclusion, comparison of four WGA methods in terms of their usefulness for microarray assays to detect infectious agents revealed that the Phi29 v2 and OmniPlex WGA methods were superior with regard to the quantity and quality of DNA, suggesting that these particular DNA amplification methods might facilitate the identification of unknown pathogens from small samples.

ACKNOWLEDGMENTS

We thank Dr. Imaoka and Dr. Okutani for various help.

This study was supported by the Health Sciences and Labour Research Grants from the Ministry of Health, Labour and Welfare of Japan.

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