JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1984, p. 608–613
0095-1137/84/100608-06$02.00/0

Vol. 20, No. 4

Rapid Procedure for Isolation of Plasmid DNA and Application to Epidemiological Analysis

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Received 27 January 1984/Accepted 18 June 1984

A rapid and simple plasmid isolation procedure was developed for the epidemiological analysis of plasmid-mediated antimicrobial resistance. By this method, plasmid DNAs ranging in molecular weight between 2.0 and 122 × 10^6 could be detected. Various bacteria, such as strains of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Staphylococcus aureus*, could be analyzed. The plasmid DNA obtained could be directly used for restriction endonuclease analysis without further purification. In addition, this method made it possible to analyze several cultures at the same time.

Epidemiological investigation of antibiotic-resistant bacteria employs techniques such as restriction endonuclease digestion of plasmid DNA in addition to the analysis of resistance markers or incompatibilities. Recent studies indicate that certain plasmids become prevalent in addition to certain antibiotic-resistant bacteria (11, 13–15, 17). Application for epidemiological analysis, however, involves centrifugation in cesium chloride-ethidium bromide density gradients for plasmid DNA purification. It is also hampered by our inability to treat many isolates at the same time. Therefore, analysis is performed only with representative strains. As a result, the relation between these selected strains and their epidemiological background is not clear. A rapid isolation procedure of plasmid DNAs useful in epidemiological analysis should satisfy the following four conditions: (i) plasmid DNA obtained can be directly used for restriction endonuclease analysis; (ii) large and small plasmid DNAs are detectable; (iii) it is applicable to a variety of bacteria, such as members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Staphylococcus aureus*; (iv) a large number of bacterial cultures can be examined at the same time. Recently, rapid isolation procedures that allow plasmid DNA material to be used directly in restriction endonuclease analysis have been reported (2, 5, 8, 9). We did not find them suitable for epidemiological investigation. For instance, treatment with lysozyme (2, 9) and RNase (2, 5, 9) or with heat (8) seemed unnecessary. The procedure with phenol (8, 9) required distillation and ether extraction of phenol to avoid interference with the endonuclease reaction. To obtain reproducible results, we did not consider removal of impurities and control of salt concentration in these methods.

We sought to develop a method that satisfied the above four conditions by adopting two previously described methods (2, 8).

MATERIALS AND METHODS

Chemicals, reagents, and equipment. Phage λ DNA and restriction enzymes of *Eco*RI and *Bam*HI were purchased from Takara Shuzo Co., Japan.

The lysostaphin solution consisted of 12 U of lysostaphin (Sigma Chemical Co., St. Louis, Mo.) per ml, 100 mM NaCl, 40 mM Tris-NaOH, and 50 mM disodium EDTA (pH 6.9). This solution was stored at −20°C. Lysing solution for strains of the family *Enterobacteriaceae* and of *S. aureus* was prepared as follows: 4% sodium dodecyl sulfate (SDS)–100 mM Tris was stock solution and was stored at room temperature; for use, an equal volume of freshly prepared 0.4 N NaOH was added. Lysing solution for *Pseudomonas aeruginosa* and *H. influenzae* was identical to that of *Enterobacteriaceae* and *S. aureus* except that the concentration of NaOH was 0.16 N. Buffer A consisted of stock buffer solution containing 400 mM Tris-acetic acid and 20 mM disodium EDTA (pH 8.0), which was diluted 10 times with distilled water for use. Buffer B consisted of 3 M sodium acetate-acetic acid (pH 5.5). Buffer C consisted of 10 mM Tris-acetic acid and 2 mM disodium EDTA (pH 8.0). Buffer D consisted of 1 M sodium acetate, 10 mM Tris-acetic acid, and 2 mM disodium EDTA (pH 8.0). All buffers were autoclaved at 115°C for 10 min and stored at 4°C.

Polypropylene centrifuge tubes (1.5 ml) with caps (Eppendorf type) and polypropylene micropipette tips (Excel type) were used after autoclaving at 115°C for 10 min and drying.

Bacterial strains and plasmids. Plasmids of known molecular weights used as controls are listed in Table 1. Clinical isolates included the following: *Escherichia coli* CU06711, CU18352, and CU09232; *Citrobacter freundii* CU17160 and CU13222; *Citrobacter diversus* CU18752; *Enterobacter cloacae* CU06407 and CU13592; *Enterobacter aerogenes* CU13116; *Serratia marcescens* CU16741; CU07392, and CU08352; *Klebsiella pneumoniae* CU03092 and CU06351; *Klebsiella oxytoca* CU19232; *Proteus vulgaris* CU08806; *Providencia rettgeri* CU21322; *Morganella morganii* CU23232; *Pseudomonas aeruginosa* CU13325, CU20304, and CU18262; *H. influenzae* FM0634, FM0595, and FM0626; and *Staphylococcus aureus* FM0333, FM0365, and FM0371.

Growth media. Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) was employed for culturing all strains. For *H. influenzae*, a strip of X-V factor (BBL Microbiology Systems, Cockeysville, Md.) was added per 3 ml of broth. Bacteria were grown in 3 ml of broth at 37°C for 16 to 18 h.

Plasmid DNA isolation procedure. Cultures were harvested by centrifugation at room temperature at 2,190 × *g* (3,600 rpm) for 5 min. Subsequent centrifugation was performed as

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TABLE 1. Plasmids and their host strains

Host strain and plasmid	Mol wt ($\times 10^6$)	Reference(s)
Escherichia coli K-12 C600		
R746	122	6
RA1	86	6
R222	70	6
RP4	36	6
Rms149	36	4
R46	32	3, 6
RSa	23	6
Col E1	4.2	1
Pseudomonas aeruginosa PAO1		
RP4	36	6, 7

described except for temperature adjustments. Either 200 μl of buffer A or 200 μl of lysostaphin solution, respectively, was added to the cell pellet, depending on whether gram-negative bacteria or *Staphylococcus aureus* were involved. After the cell pellet was thoroughly suspended, the cell suspension was transferred to a polypropylene centrifuge tube. For analysis of *Staphylococcus aureus*, the tube was further incubated at 37°C for 10 min. Lysing solution (400 μl) was added to the cell suspension; then the tube was gently inverted 5 to 10 times and allowed to stand at room temperature for 5 min. For neutralization, 300 μl of cold (4°C) buffer B was added and gently mixed by inversion 10 to 20 times. After being maintained at 0°C for 5 min, the tube was centrifuged at room temperature and then again maintained at 0°C for 10 min. Salt-precipitated material was centrifuged at 0°C, and the supernatant was transferred to another tube by decantation (ca. 700 μl). An equal volume of chloroform was added and emulsified by inversion 5 to 10 times, followed by centrifugation at 0°C to break the emulsion. Of the upper aqueous phase, 500 μl was carefully transferred to another tube with a polypropylene micropipette tip, which was cut to expand to more than 5 mm in diameter. Cold (−20°C) ethanol (1 ml) was added and inverted 5 to 10 times. The tube was maintained at 0°C for 5 min. The precipitate was collected by centrifugation at 0°C, and the supernatant was removed by decantation. The pellet was dissolved in 100 μl of buffer C. At this time, the total volume was about 130 μl. This plasmid DNA solution was subjected to electrophoresis according to the method mentioned below for molecular weight estimation, or the solution was stored at −20°C until use.

Restriction endonuclease analysis and transformation. Buffer C (350 μl) and 50 μl of buffer D were added to about 100 μl of the plasmid DNA solution that remained after the molecular weight determination, and this solution was mixed. The solution was ethanol precipitated as described above. The supernatant was completely removed by decantation. The pellet was dried by evaporation under a vacuum (about −730 mm Hg [−97.325 kPa]) and dissolved in 40 μl of autoclaved water. For restriction enzyme analysis, 10 μl of 5×-concentrated restriction endonuclease assay buffer and 5 U of endonuclease solution were added to the material and allowed to react for 3 h. The assay buffer used and the reaction temperature were according to the instructions of the manufacturer. After digestion, the sample was subjected to electrophoresis as described below. For transformation, 10 μl of 200 mM Tris-acetic acid–10 mM disodium EDTA–200 mM NaCl (pH 8.0) was added and used as previously described (10).

Agarose gel electrophoresis. Electrophoresis was carried out on a horizontal apparatus. Either 0.7 or 1.0% agarose gels were used depending on the use of whole plasmid DNA or restriction endonuclease-digested fragments, respectively. Agarose was melted in buffer A by heating at 115°C for 5 min in an autoclave, then cooled to 60°C, and poured into the apparatus. Usually, 25 μl of sample was mixed with 5 μl of tracking dye solution, composed of 0.1% bromocresol purple and 50% glycerol in water, and applied to the wells. After being electrified for 5 min, buffer A was poured over the gel to a thickness of 2 mm. Electrophoresis was performed at 10 V/cm for 2 h. Then the gel was stained with 0.5 μg of ethidium bromide solution per ml for 1 h. A photograph of the stained gel was taken with a shortwave UV transilluminator.

RESULTS AND DISCUSSION

Cell lysis and removal of chromosomal DNA and RNA. Vinograd and Lebowitz (19) report that, under alkaline conditions (pH 11.5 to 12.3), covalently closed circular DNA is not affected, whereas linear DNA is denatured. Rapid isolation techniques so far reported (2, 5, 8), which make it possible to use plasmid DNA material obtained directly in restriction endonuclease analysis without the cesium chloride-ethidium bromide density equilibrium centrifugation purifying process, utilize such physical properties to denature linear chromosomal DNA. We prepared lysing solution that had five different alkaline conditions by adding an equal volume each of 0.08, 0.16, 0.24, 0.32, and 0.4 N NaOH to 4% SDS–100 mM Tris. Under these conditions, we tried to extract plasmid DNA of various species and strains to investigate cell lysis and the removal of chromosomal DNA and RNA. The result of extracting plasmid DNA from *Escherichia coli* K-12 strain ML1410 harboring pCU81U09

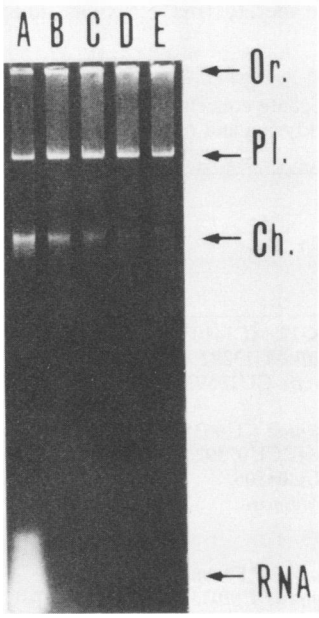

FIG. 1. Effect of alkali treatment to eliminate chromosomal DNA and RNA. Cell suspensions (200 μl) of *Escherichia coli* K-12 ML1410 harboring pCU81U09 (120 × 10^6) in buffer A were lysed by adding five kinds of lysing solutions (400 μl) shown below; each equal volume of 0.08 (lane A), 0.16 (lane B), 0.24 (lane C), 0.32 (lane D), and 0.4 (lane E) N NaOH was added to 4% SDS–100 mM Tris. Or., Origin of electrophoresis; Pl., plasmid DNA; Ch., chromosomal DNA.

of 120×10^6 in molecular weight which was transferred from *C. freundii* CU13222 is shown in Fig. 1. All pH conditions used allowed cell lysis as soon as the solution was added, and all samples became clear and viscous. When 0.08 N NaOH was added, chromosomal DNA and RNA were observed in addition to plasmid DNA (Fig. 1, lane A). In proportion to the increase in the concentration of NaOH, RNA was completely removed, and chromosomal DNA was decreased, too (lanes B, C, and D). In the case of 0.4 N NaOH, nothing but plasmid DNA remained, whereas chromosomal DNA was not detected (lane E). Concerning various bacterial strains, adequate concentrations of NaOH which should be added to lysing solution are listed in Table 2. Under these conditions, cells of each bacterium except *Staphylococcus aureus* were sufficiently lysed only with alkaline SDS treatment, and plasmid DNA material freed from chromosomal DNA could be obtained. On the other hand, cell lysis of *Staphylococcus aureus* required lysostaphin before SDS-alkali treatment. This procedure also yielded plasmid DNA that was free of chromosomal DNA. In any case, only plasmid-DNA-containing samples were obtained without lysozyme and SDS-alkali combination treatment (2) or without heating of a cell-lysed solution (8). Also, RNase treatment (2, 5, 9) was found to be unnecessary since RNA was removed together with chromosomal DNA.

Removal of impurities. To remove impurities from cell-lysed solution, techniques such as salting out (2, 5), phenol treatment (9), or phenol-chloroform extraction (8) have been established. It was found that phenol with or without chloroform is most efficient for the removal of protein-chromosomal DNA complexes from cell-lysed material. However, these techniques were not suitable since SDS remaining in the supernatant had considerable influence on the endonuclease reaction. Salting out was also unsuitable. Consequently, we designed the following procedure: a half volume of 3 M sodium acetate (pH 5.5) was added to cell-lysed solution for salting out and neutralization (neutralization was reproducibly performed at pH 5.4 to 5.6); then an equal volume of chloroform was added to the supernatant obtained by centrifugation, mixed, and centrifuged to remove the white flocculations. The advantage of this procedure was that it avoided complicated steps such as ether extraction of phenol. All floating white material remaining in the supernatant after salting out could be removed with this procedure. Moreover, by a 5-min precentrifugation of the neutralized mixture, large accumulations of flocculated material could be removed at this junctive. This process became a key step for a greater yield of supernatant when the neutralized mixture was centrifuged after salting out for 10 min at 0°C.

TABLE 2. Adequate concentrations of NaOH added to lysing solutions for cell lysing and removal of chromosomal DNA and RNA

Strain	Adequate concn of NaOH (N)[a]	pH of lysing solution[b]	pH of lysate[b,c]
Escherichia coli K-12 ML1410[d]	0.4	12.97–12.99	12.80–12.82
Citrobacter freundii CU13222	0.4		
Enterobacter cloacae CU13592	0.4		
Klebsiella pneumoniae CU06351	0.4		
Serratia marcescens CU07392	0.4		
Proteus vulgaris CU08806	0.4		
Pseudomonas aeruginosa PAO1[e]	0.16	12.65–12.69	12.44–12.46
Haemophilus influenzae FM0634	0.16		
Staphylococcus aureus FM0333[f]	0.4	12.97–12.99	12.79–12.82

[a] Each lysing solution was prepared by adding an equal volume of this concentration of NaOH to 4% SDS–100 mM Tris solution.
[b] Measurement of pH value was carried out by using a glass electrode pH meter.
[c] Lysate was the mixture of one volume of cell suspension and two volumes of lysing solution.
[d] Harboring pCU81U09.
[e] Harboring RP4.
[f] Cells were treated with lysostaphin before lysing solution was added.

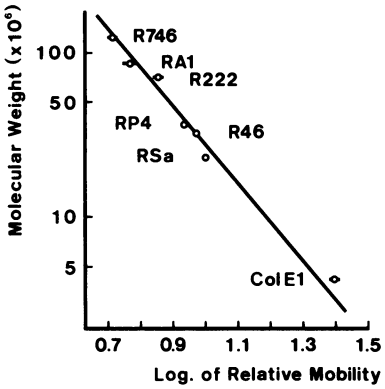

FIG. 2. Correlation of molecular weights to relative mobilities for seven plasmid DNAs of known molecular weights extracted from *Escherichia coli* K-12 C600. The relative mobilities of the other six plasmids were calculated with the mobility of the plasmids, RSa as 10, and the logarithm of each was plotted.

Molecular weight estimation. The correlation of molecular weights to relative mobilities obtained by our method is shown in Fig. 2. Of the known molecular weight plasmids, seven kinds harbored in *E. coli* K-12 strain C600 were used: R746, RA1, R222, RP4, R46, RSa, and ColE1. Based on the results of electrophoresis of extracted plasmid DNA, which was carried out five times on all seven plasmids, the equation for the regression line was obtained by a least-squares method. Relative mobilities were correlated to known molecular weights.

Restriction endonuclease analysis. The use of restriction endonucleases requires adequate salt concentrations that vary from less than 20 mM to greater than 100 mM of Na^+. Measurement of the Na^+ concentrations of the samples, which were chloroform treated followed by ethanol precipitation and solution in 100 μl of buffer C, yielded between 70 and 150 mM repeatedly in a flame photometer. This range was considered unsuitable for direct restriction endonuclease analysis. By adding 350 μl of buffer C and 50 μl of buffer D, we controlled Na^+ concentration of the sample to 100 to 120 mM. This material was ethanol reprecipitated, the supernatant was decanted as completely as possible, and the ethanol remaining in the pellet was dried completely by evaporation. The Na^+ concentration, when the pellet was dissolved in 40 μl of distilled water, was less than 20 mM and suitable for direct use in restriction endonuclease analysis. An example of restriction endonuclease fragmentation patterns of Rms149 and R46 is shown in Fig. 3. *Eco*RI fragments of phage λ DNA (Fig. 3, lane D) served as a standard (16), and estimation of molecular weights was performed with regard to each fragment. The molecular weights of produced

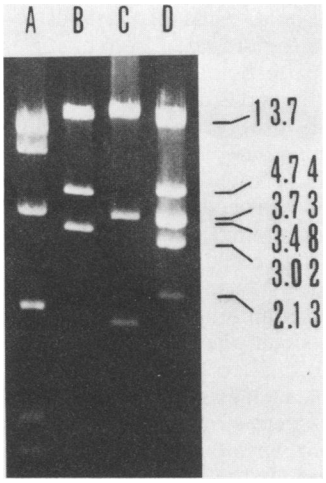

FIG. 3. Restriction endonuclease digests of plasmids Rms149 and R46. Lanes: A, *Eco*RI digests of Rms149; B, *Eco*RI; C, *Bam*HI digests of R46; D, *Eco*RI digests of phage λ DNA used as molecular weight standards. Numbers indicate molecular weights ($\times 10^6$).

*Eco*RI fragments of Rms149 (Fig. 3, lane A) were estimated at 11.5×10^6, 8.2×10^6, 3.9×10^6, 2.0×10^6, 1.2×10^6, and 1.0×10^6 from the top of the figure. Hedges and Jacoby (4) describe the molecular weights of *Eco*RI fragments of Rms149 to be 12.0×10^6, 8.4×10^6, 4.0×10^6, and 2.0×10^6. Our experimental results agreed except for newly confirmed fragments with molecular weights of 1.2×10^6 and 1.0×10^6. In the case of R46, the molecular weights of *Eco*RI fragments (Fig. 3, lane B) were 22.7×10^6, 4.8×10^6, 3.4×10^6, and 1.0×10^6, and those of *Bam*HI fragments (Fig. 3, lane C) were 26.5×10^6, 3.7×10^6, and 1.8×10^6. The restriction endonuclease cleavage map of R46 presented by Brown and Willetts (3) shows the molecular weights of *Eco*RI fragments to be 22.65×10^6, 4.62×10^6, 3.44×10^6, 1.02×10^6, and 0.27×10^6 and the molecular weights of *Bam*HI to be 26.52×10^6, 3.56×10^6, and 1.92×10^6. We had the same results except that the smallest fragment (0.27×10^6) of *Eco*RI digest was not observed.

Transformation. Plasmid DNA extracts of RSa, R46, Rms149, and RP4 were used as the donor DNAs, and *Escherichia coli* K-12 C600 was used as the recipient. A total of 10^4 cells of transformants were obtained.

Application to clinical isolates of various species. The molecular weights of plasmids isolated from various clinical isolates are presented in Table 3, and the electrophoretic patterns of their plasmids are shown in Fig. 4 and 5. Plasmids were successfully isolated from all strains of *Enterobacteriaceae*, *H. influenzae* and *Staphylococcus aureus*. Especially, as shown in lanes G and H in Fig. 5, two plasmids of about 20×10^6 would not have been detected in the presence of chromosomal DNA. Plasmids from *Pseudomonas aeruginosa* (CU13325 and CU20304) were detected. However, complete removal of their chromosomal DNAs was not achieved even when the limits of the pH range for plasmid DNA were applied. In the case of *Pseudomonas aeruginosa* CU18262, plasmids were not detected, although the strain was resistant to tobramycin and the existence of plasmids was strongly suspected. The molecular weights of all plasmids isolated from various clinical isolates were similar to those reported previously for *Enterobacteriaceae* (6), *H. influenzae* (12), *Staphylococcus aureus* (18), and *Pseudomonas aeruginosa* (7). Lanes E to J in Fig. 5 show plasmids from various species of gentamicin-resistant bacteria isolated in our hospital. All isolates harbored plasmids of the same molecular weight, 100×10^6. From transconjugants obtained by mating these resistant strains with *Escherichia coli* K-12 W3104,

TABLE 3. Molecular weights of plasmids detected from various clinical isolates

Strain tested	Mol wts of plasmids detected ($\times 10^6$)
Escherichia coli CU06711	83, 44
E. coli CU18352	59, 32, 2.9
E. coli CU09232	72, 42, 2.7
Citrobacter freundii CU17160	100, 3.9, 2.0
C. freundii CU13222	120, 48
C. diversus CU18752	68, 45, 3.5
Enterobacter cloacae CU06407	100, 83, 48, 2.6, 2.0
E. cloacae CU13592	110, 65
E. aerogenes CU13116	120, 100, 21, 2.7
Serratia marcescens CU16741	100, 63, 42, 20, 2.3
S. marcescens CU07392	55, 48, 2.3
S. marcescens CU08352	32, 25
Klebsiella pneumoniae CU03092	100, 68
K. pneumoniae CU06351	60, 32, 2.5
K. oxytoca CU19232	72, 43
Proteus vulgaris CU08806	100, 83, 2.6, 2.0
Morganella morganii CU23232	36, 28
Providencia rettgeri CU21322	115, 90
Pseudomonas aeruginosa CU13325	85, 45, 3.5, 3.3
P. aeruginosa CU20304	117, 54, 16
P. aeruginosa CU18262	ND[a]
Haemophilus influenzae FM0634	43
H. influenzae FM0595	43
H. influenzae FM0626	38
Staphylococcus aureus FM0333	38, 11, 2.1
S. aureus FM0365	37, 10.5
S. aureus FM0371	17

[a] ND, Not detected.

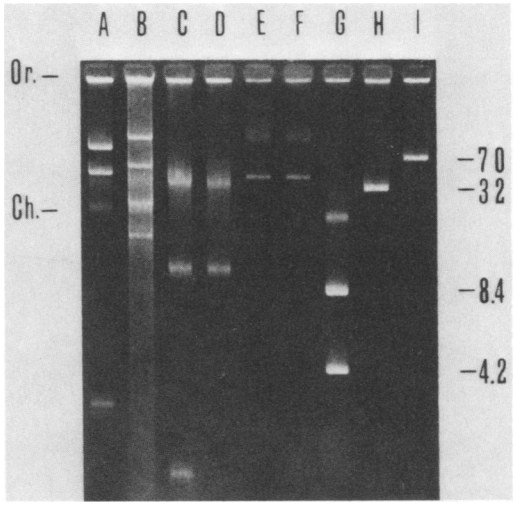

FIG. 4. Agarose gel electrophoretogram of plasmids extracted from various species of clinical isolates. Lanes: A and B, *Pseudomonas aeruginosa* CU13325 and CU20304, respectively; C and D, *Staphylococcus aureus* FM0333 and FM0365, respectively; E and F, *H. influenzae* FM0634 and FM0595, respectively; G, dimer (middle band) and monomer (bottom band) of ColE1; H and I, R46 and R222, respectively, used as molecular weight standards. Numbers indicate molecular weights ($\times 10^6$). Or., Origin of electrophoresis; Ch., chromosomal DNA.

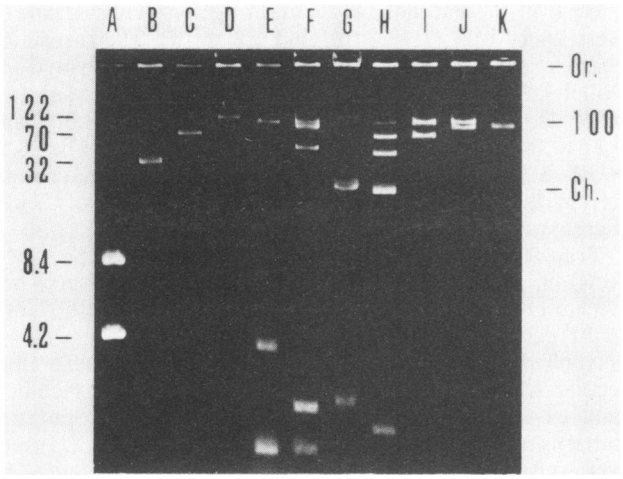

FIG. 5. Agarose gel electrophoretogram of plasmids extracted from clinical isolates of the family *Enterobacteriaceae*. Lanes: A, dimer (middle band) and monomer (bottom band) of ColE1; B, C, and D, R46, R222, and R746, respectively, used as molecular weight standards; E, *Citrobacter freundii* CU17160; F, *Enterobacter cloacae* CU06407; G, *Enterobacter aerogenes* CU13116; H, *Serratia marcescens* CU16741; I, *Klebsiella pneumoniae* CU03092; J, *Proteus vulgaris* CU08806; K, *Escherichia coli* CU06711. The region from lanes E to J contains plasmids of the same molecular weight, 100×10^6. Numbers indicate molecular weights ($\times 10^6$). Or., Origin of electrophoresis; Ch., chromosomal DNA.

plasmids of 100×10^6 were isolated and digested with *Eco*RI by our method. The result of *Eco*RI digestion is shown in Fig. 6. The identity of plasmids from different species was demonstrated with patterns they showed after endonuclease treatment (Takahashi and Nagano, in press).

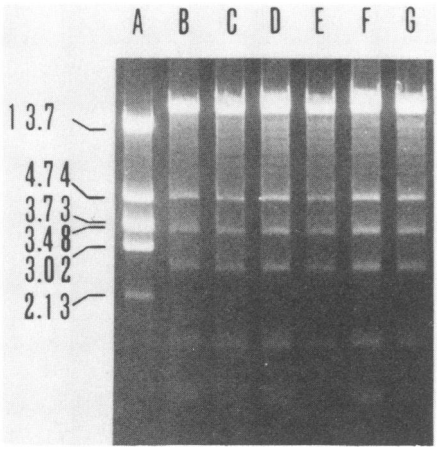

FIG. 6. *Eco*RI restriction endonuclease digests of plasmids of the same molecular weight (100×10^6) extracted from *Escherichia coli* K-12 W3104 transconjugants obtained by mating with various clinical isolates of *Enterobacteriaceae*. Lanes: A, *Eco*RI digests of phage λ DNA used as molecular weight standards; B, derived from *Citrobacter freundii* CU17160; C, *Enterobacter cloacae* CU06407; D, *Enterobacter aerogenes* CU13116; E, *Serratia marcescens* CU16741; F, *Klebsiella pneumoniae* CU03092; G, *Proteus vulgaris* CU08806. All plasmids contain common molecular weight fragments of 86×10^6, 5.0×10^6, 3.5×10^6, 2.7×10^6, 1.6×10^6, and 1.2×10^6. Numbers indicate molecular weights ($\times 10^6$).

Stability of plasmids isolated. Plasmids R746, R222, R46, and Col E1 were each isolated from host *E. coli* K-12 strain C600, and the patterns of these plasmids were compared when first isolated and after 6 months of storage at −20°C. No changes were observed in their mobilities or luminous grade, and plasmid samples obtained by our method were found to be stable enough for long-term preservation.

Conclusion. Our procedure satisfied the four conditions required for epidemiological analysis. We obtained plasmid DNA material that could be directly used for restriction endonuclease analysis without the cesium chloride-ethidium bromide density equilibrium centrifugation purifying process. Large and small plasmid DNAs ranging in molecular weight from 2.0×10^6 to 122×10^6 were detected. Various bacterial species, such as members of the family *Enterobacteriaceae*, and species of *Pseudomonas aeruginosa*, *H. influenzae*, and *Staphylococcus aureus* could be analyzed by the same method. It took 60 min to obtain plasmid DNA material for molecular weight determination and an additional 20 min to allow the material to be used for restriction endonuclease analysis. Furthermore, since the procedure was technically simple, a large number of bacterial cultures could be analyzed simultaneously.

ACKNOWLEDGMENTS

We are grateful to T. Sawai of the Faculty of Pharmaceutical Science, Chiba University, for his kind comments and for supplying bacterial strains and plasmids. We also thank S. Furiya and H. Kanno of Chiba University Hospital, Chiba University School of Medicine, for their kind comments.

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