Tohoku J. Exp. Med., 2001, **194**, 205-212

Enterobacterial Repetitive Intergenic Consensus Sequence-Based PCR (ERIC-PCR); its Ability to Differentiate *Streptococcus pyogenes* Strains and Applicability to the Study of Outbreaks of Streptococcal Infection

MASAKADO MATSUMOTO, YASUMOTO SUZUKI, YUTAKA MIYAZAKI, DAISUKE TANAKA,[1] TOMIHISA YASUOKA,[2] KYOKO MASHIKO,[3] RYO-ICHI ISHIKITA[4] and JUN-ICHI BABA[4]

Department of Microbiology, Aichi Prefectural Institute of Public Health, Nagoya 462-8576,
[1]*Department of Bacteriology, Toyama Institute of Health, Toyama 939-0363,*
[2]*Division of Infectious Diseases, Public Health Institute of Kochi Prefecture, Kochi 780-0850,*
[3]*Department of Microbiology, Ibaraki Prefectural Institute of Public Health, Ibaraki 310-0852, and*
[4]*Department of Microbiology, Fukuoka City Institute of Hygiene and Environment, Fukuoka 810-0065*

MATSUMOTO, M., SUZUKI, Y., MIYAZAKI, Y., TANAKA, D., YASUOKA, T., MASHIKO, K., ISHIKITA, R. and BABA, J. *Enterobacterial Repetitive Intergenic Consensus Sequence-Based PCR (ERIC-PCR); its Ability to Differentiate Streptococcus pyogenes Strains and Applicability to the Study of Outbreaks of Streptococcal Infection.* Tohoku J. Exp. Med., 2001, **194** (4), 205-212 —— We evaluated the ability of enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR) to differentiate 95 *Streptococcus pyogenes* strains with M or T serotypes isolated from sporadic streptococcal infections as compared with M or T serotypings and pulsed-field gel electrophoresis (PFGE). Although the ERIC-PCR had less discriminatory power, defined as the ability to divide the strains with the same serotypes into the different sub-types, than PFGE, it consistently classified the strains into 16 patterns with a high correlation with M or T serotyping. The PCR method further discriminated 4 M or T serotypes into sub-types. The application of ERIC-PCR to 5 outbreaks of streptococcal infection produced the results that agreed closely with those of T serotyping and PFGE. ERIC-PCR has sufficient discriminatory power and is a quick and relatively easy technique, making it useful for routine epidemiological investigations. ———— enterobacter-

Received March 23, 2001; revision accepted for publication August 13, 2001.

Address for reprints: Masakado Matsumoto, Ph.D., Department of Microbiology, Aichi Prefectural Institute of Public Health, Nagoya 462-8576, Japan.
e-mail: masakado_matsumoto@mail.pref.aichi.jp

ial repetitive intergenic consensus sequence (ERIC); PCR; *Streptococcus pyogene*
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Streptococcus pyogenes is a medically important bacterial pathogen known to cause variety of systemic and cutaneous infection, and it is the most common cause of acute pharyngitis, and, in some cases causes fatal bacteremia and streptococcal toxic shock syndrome (Strep TSS). Outbreaks of streptococcal infection occur occasionally in both health care setting and community (Gunzenhauser et al. 1995; Gruteke et al. 1996). Food-borne outbreaks were recently reported in developped countries (Yamamoto et al. 1998; Matsumoto et al. 1999). Thus, *S. pyogenes* remains a public health problem in many countries, necessitating appropriate typing procedures for epidemiological investigations.

Over more than half a century, serological analysis using M and T antigens has been established as a gold standard for traditional typing procedures (Moody et al. 1965; Rotta et al. 1971). However, these methods have limitations. It has been reported that many strains of *S. pyogenes* can not be typed with the available M antisera and that T serotyping lacks specificity. In the present study, we applied enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR) to the typing of 95 strains of *S. pyogenes* from sporadic streptococcal infections in order to overcome the limitations and drawbacks of M or T serotyping. The results of typing using ERIC-PCR and those using plused-field gel electrophoresis (PFGE) were compared with the results of the M or T serotypings to evaluate the ability to differentiate the strains of *S. pyogenes*. In addition, we analyzed 43 strains of *S. pyogenes* from 5 outbreaks of streptococcal infection using ERIC-PCR and PFGE to further evaluate the applicability to epidemiological investigations of *S. pyogenes*.

MATERIALS AND METHODS

Bacterial strains

Of 138 strains of *S. pyogenes* used in this study, 95 were isolated from sporadic cases involving 14 patients with Strep TSSs and 81 with mild streptococcal infections like pharyngitis and tympanitis in 1984–1998 in Japan. Among the 95 strains, 58 were isolated in a general hospital in Toyama City, Toyama Prefecture and belonged to 9 different T serotypes. Nineteen strains with 4 M and T serotypes from 14 Strep TSSs and 5 mild streptococcal infections, which were isolated around Japan, were stocked at the College of Health Professions, Toho University, Tokyo. The other 18 strains with 7 T serotypes were isolated in 3 general hospitals in Aichi Prefecture. M serotyping of 19 strains at Toho University had been performed by standard methods (Rotta et al. 1971). T serotyping of all 95 strains was performed using a commercially available streptococcal grouping kit and group A, T typing sera (Denka Seiken Co., Ltd., Tokyo).

Forty-three strains of *S. pyogenes* were isolated from 5 outbreaks of streptococcal infection during 1996–1998 in Japan. Of the 5 outbreaks, those in Aichi, Ibaraki, and Fukuoka were food-borne. M serotyping for 10 strains from Toyama and T serotyping for all 43 strains were performed as described above.

ERIC-PCR

Total genomic DNA was extracted from bacterial cells using InstaGene TM Matrix (Bio-Rad Laboratories, Hercules, CA, USA). DNA concentrations were determined with the Genequant RNA/DNA calculator (Pharmacia, Uppsala, Sweden) and adjusted to 25 ng/μl using sterilized distilled water. Primers used for ERIC-PCR were ERIC1R (5′-ATGTAAGCTC-

CTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Versalovic et al. 1991).

Amplification was conducted in a 50-μl reaction mixture containing a dNTP mixture (with a final concentration of 0.25 mM dATP, dTTP, dCTP, and dGTP), 10-fold concentrated *Ex Taq* buffer (final concentration of 2.0 mM Mg^{2+}), 1.5 U of TaKaRa *Ex Taq* (Takara Shuzo, Kyoto), 50 pmol of the primer, and 100 ng of genomic DNA.

Samples were amplified on a Perkin Elmer GeneAmp PCR System 9600 (Perkin Elmer Japan, Chiba) as follows; 40 cycles of 94°C for 1 minute, 25°C for 1 minute, and 74°C for 4 minutes (Gruteke et al. 1996). After the amplification, 5 μl of the reaction mixture was run in a 1.5% agarose gel (Wako Pure Chemical Industries Ltd., Osaka) and the gel stained with ethidium bromide. Lambda phage DNA/Hind III digest (Nippon Gene, Tokyo) and a 100-base ladder marker (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used as DNA molecular weight markers. The band patterns of all lanes were visually compared. The intensity of the amplified bands was also considered when differentiating between strains. Strains showing differences of one or more bands were considered to be different ERIC types and assigned the capital letter E followed by a number.

PFGE

Preparation of DNA for restriction endonuclease digestion and subsequent analysis by PFGE was done according to the method of Nakashima et al. (1997). Briefly, the chromosomal DNA was digested with the restriction endonuclease *Sma*I and then DNA fragments were separated using a LKB2015 Pulsaphor (Pharmacia, Uppsala, Sweden) for 20 hours at 180 V with pulse times ranging from 1 to 40 seconds. Lambda DNA concatemers (Amersham Pharmacia Biotech) were used as size markers. DNA fragment patterns were assessed visually and the interpretation of clonal relatedness was based on that recently proposed by Tenover et al. (1995). Isolates were considered to differ in PFGE type, if there was a difference of more than four bands (Tenover's category: possibly related and different). The different PFGE types were assigned a number following the letter P. Isolates were considered to differ in sub-type within the same PFGE type if there was a difference of up to three DNA bands and thus allocated a decimal numeral following the same PFGE type number.

RESULTS

Differentiation of 95 S. pyogenes strains from sporadic streptococcal infections using ERIC-PCR and PFGE

In a preliminary series of experiments for the analysis of 11 strains with 11 different serotypes by ERIC-PCR using ERIC1R and ERIC2 primers, we found that the ERIC2 primer was more discriminatory than the ERIC1R primer. Accordingly, we used ERIC2 for ERIC-PCR in this study. With the ERIC-PCR method, the *S. pyogenes* strains yielded 4 to 9 major PCR products with molecular size of between approximately 0.28 kb and 2.7 kb with various band intensities. ERIC-PCR amplification of genomic DNA of the 95 strains generated 16 different patterns (E1-E16) from 11 distinctive M or T serotyped strains commonly encountered in clinical laboratories (Table 1). The E5 type shared serotypes 6, 22 and 28. E7 was classified as serotypes 11 and 28, but the other 14 ERIC types were M or T serotype-specific. Moreover, the PCR method could differentiate the strains belonging to serotypes 11, 18, 28 and T-B3264 into 2 or 4 sub-types.

We examined the intensity of the amplified bands of agarose gel electrophoresis to confirm reproducibility of the faint band(s). E1 was similar to E3, but the intensity of the band estimated to be 1.3 kb was different between them (Fig. 1, lanes 1 and 3). Although the

TABLE 1. *Results of ERIC-PCR and PFGE typing of 95 S. pyogenes strains from sporadic streptococcal infections*

M or T serotype[a]	Number of strains	ERIC-PCR[b]	PFGE[b,c]
1	10	E1 (10)	P1(10)
2	10	E2 (10)	P2(5), P3(3), P4(1), P5(1)
3	10	E3 (10)	P6(9), P7(1)
4	10	E4 (10)	P8(9), P9(1)
6	8	E5 (8)	P10(8)
11	7	E6 (4)	P11(3), P12(1)
		E7 (3)	P13(3)
12	10	E8 (10)	P14(9), P15(1)
18	5	E9 (4)	P16(3), P17(1)
		E10 (1)	P18(1)
22	5	E5 (5)	P19(4), P20(1)
28	10	E11 (6)	P21(6)
		E12 (1)	P22(1)
		E5 (1)	P21(1)
		E7 (2)	P23(1), P24(1)
T-B3264	10	E13 (7)	P25(7)
		E14 (1)	P25(1)
		E15 (1)	P26(1)
		E16 (1)	P25(1)
Total	95		

[a]Results of M serotyping for 19 strains were identical to those of T serotyping.
[b]The number of strains is given in parentheses.
[c]PFGE types, not sub-types, are listed.
ERIC-PCR, enterobacterial repetitive intergenic consensus sequence-based PCR; PFGE, pulsed-field gel electrophoresis.

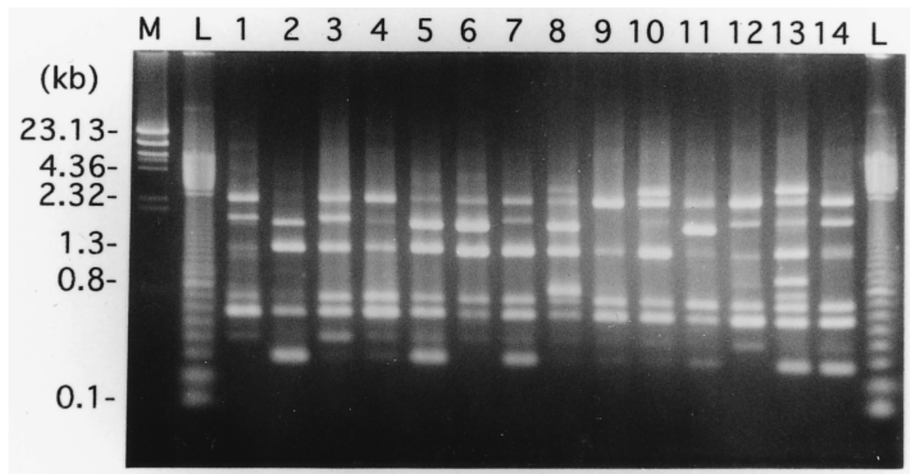

Fig. 1. Representative ERIC-PCR patterns of *S. pyogenes* strains with 11 different M or T serotypes from sporadic streptococcal infections. Lane M is a lambda marker (lambda phage DNA/Hind III digest), and lane L is a 100-base ladder marker. Lanes 1 to 11, E1 to E11; lane 12, E13; lane 13, E14; lane 14, E15.

profiles of E4 and E9 were analogous, E4 had a weak second band one position from the top, whereas E9 did not (Fig. 1, lanes 4 and 9). The profiles of E7 and E15 were also analogous, however, the intensity of the third band from the top was higher for E7 than for E15 (Fig. 1, lanes 7 and 14). Because this PCR method was performed under a low annealing temperature, we tested for the reproducibility of the band intensity of 6 ERIC types described above. As a result, identical band patterns and intensity was obtained by triplicate PCRs on 3 different days.

PFGE of chromosomal DNA from the 95 streptococcal strains with restriction enzyme *Sma* I resulted in banding patterns of 6 to 10 fragments, ranging in size approximately from 48.5 kb to 534 kb. The 95 strains were classified into 26 types (from P1 to P26) by PFGE patterns of *Sma* I digested DNAs of the strains (Table 1). Although serotypes 1 and 6 could not be differentiated into distinct PFGE types, the other 9 serotypes were subdivided into 2 to 4 PFGE types. Furthermore, 7 of the 26 PFGE types, consisted of 2 to 3 sub-types into the same PFGE types (data not shown).

The application of ERIC-PCR to 5 outbreaks of streptococcal infection

We examined 43 *S. pyogenes* strains from 5 outbreaks of streptococcal infection using ERIC-PCR and PFGE to evaluate the applicability of ERIC-PCR for routine epidemiological study (Table 2). ERIC-PCR classified the strains of 3 outbreaks, those in Aichi, Toyama, and Ibaraki, into the same ERIC types. The ERIC types of the strains from the 3 outbreaks were identical to those of the strains serotyped into 1, 12 and 22 from sporadic streptococcal infections. Seven strains from Kochi were differentiated into 2 ERIC types, E5 and E17. E5 was found among the ERIC types of the strains from sporadic cases, but E17 was unique with respect to the band pattern among sporadic and outbreak strains. All of 8 strains from Fukuoka were classified into E18 by ERIC-PCR. The ERIC type was different from the ERIC types of T-B 3264 strains and other M or T serotype strains from sporadic cases and also unique. Fig. 2 shows ERIC-PCR patterns of 3 representative strains from the 5 outbreaks. The results of PFGE were well consistent with those of ERIC-PCR.

TABLE 2. *Results of ERIC-PCR and PFGE typing of 43 S. pyogenes strains from 5 outbreaks of streptococcual infection*

Outbreak	T serotype	Number of strains	ERIC-PCR[a]	PFGE[a,b]
Aichi	1	8	E1 (8)	P1 (8)
Toyama	12[c]	10	E8 (10)	P14 (10)
Kochi	22	7	E5 (6)	P19 (6)
			E17 (1)	P27 (1)
Ibaraki	22	10	E5 (10)	P19 (10)
Fukuoka	B3264	8	E18 (8)	P28 (8)
Total		43		

[a]The number of strains is given in parentheses.
[b]PFGE types, not sub-types, are listed.
[c]M serotype was also 12.
ERIC-PCR, enterobacterial repetitive intergenic consensus sequence-based PCR;
PFGE, pulsed-field gel electrophoresis.

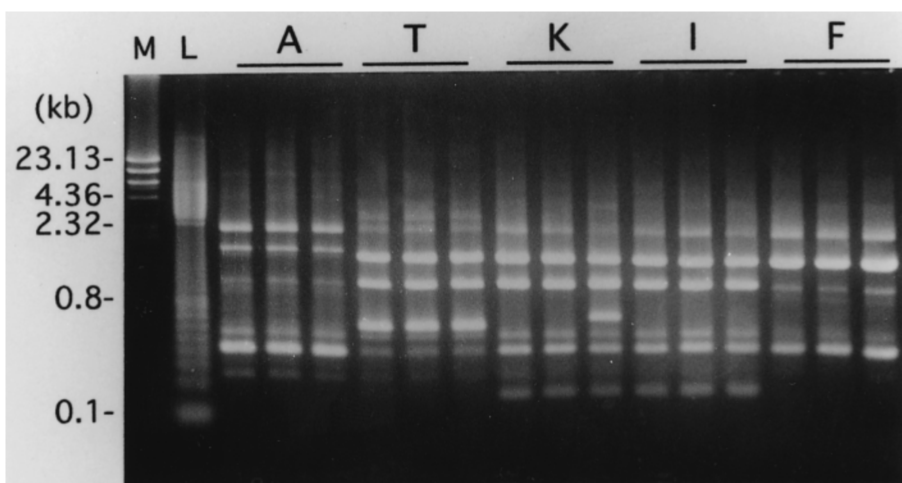

Fig. 2. ERIC-PCR patterns of *S. pyogenes* strains from 5 outbreaks of streptococcal infection. ERIC-PCR patterns of 3 representative strains in each outbreak are shown. The letters above the lanes, A, T, K, I, and F correspond to outbreaks in Aichi, Toyama, Kochi, Ibaraki, and Fukuoka. Lane M is a lambda marker (lambda phage DNA/Hind III digest), and lane L is a100-base ladder marker. ERIC-PCR types of the outbreaks A, T, I, and F are E1, E8, E5, and E18. In outbreak K, the patterns of the former 2 strains are E5 and the last one is E17.

Discussion

In typing of *S. pyogenes* using PCR-based methods, random amplified polymorphic DNA-PCR and PCR-RFLP (i.e., restriction fragment length polymorphisms of PCR-amplified products targeting to regions for streptococcal virulence factors) have been reported to be useful for epidemiological investigations (Gardiner and Sriprakash 1996; Bert et al. 1997). We applied ERIC-PCR to 95 *S. pyogenes* strains of 11distinctive M or T serotypes, since the method has apparently not been used extensively to examine group A streptococci.

ERIC sequences are repetitive elements of 126 bp which contain a highly conserved central inverted repeat, the function of which remains to be elucidated (Versalovic et al. 1991). Gram-negative bacteria have ERIC sequences, and ERIC-PCR with primers based on ERIC sequences has been successfully used to differentiate bacterial pathogens like *Shigella sonnei* and *Burkholderia* (*Pseudomonas*) *cepacia* (Liu et al. 1995a, b). The procedure is useful for typing strains and has a level of discrimination equivalent to that of PFGE. On the other hand, the presence of ERIC sequences has not been demonstrated in gram-positive bacteria yet. Struelens et al. (1993) used this fingerprinting technique to type methicillin-resistant *Staphylococcus aureus*, but the discriminatory power, defined as the ability to classify the strains with the same phenotypes into the different sub-types, was less than that of PFGE. The present study revealed that ERIC-PCR was less sensitive than PFGE in detecting the genomic diversity within *S. pyogenes* strains of the same M or T serotypes and between strains of different M or T serotypes. From the results of ours and others, the discriminatory power of ERIC-PCR on gram-positive bacteria was low, compared with that on gram-negative bacteria, possibly because of the presence or absence of ERIC sequences in bacterial genomes.

ERIC-PCR analysis of the sporadic 95 *S. pyogenes* strains with 11 serotypes demonstrated that there existed a high degree of concordance with the results obtained by ERIC-PCR and M or T serotyping. At present, M serotypings are restricted to a few reference laboratories because of a limited supply of antisera in most parts of the world including Japan, therefore, ERIC-

PCR typing of *S. pyogenes* would be applicable for M serotyping, in that it has sufficient discriminatory power and is a quick and relatively easy technique. Additionally, ERIC-PCR has the advantage over PFGE of being a faster, relatively simpler, and more applicable method for the typing of a large number of *S. pyogenes* strains in clinical laboratories. Epidemiological investigation of the 5 outbreaks of streptococcal infection revealed that the results of ERIC-PCR typing agreed closely with those of T serotyping and PFGE. The PCR method seems to be a fast, simple and inexpensive tool for routine epidemiological investigations. PFGE take a about five days to obtain the results, and the equipment is relatively expensive. In contrast, the PCR method takes only one day for to prepare and perform. Furthermore, PCR machines are available in clinical laboratories as well as public health laboratories.

As three methods, ERIC-PCR, M or T serotyping, and PFGE, evaluated had both advantages and disadvantages for the typing of *S. pyogenes* strains and thus different uses: ERIC-PCR and M or T serotyping for routine and preliminary investigations and PFGE for full investigation such as the typing of strains which could not be differentiated by the former methods.

Acknowledgments

We thank Teiko Murai (College of Health Professions, Toho University, Tokyo) for providing the *S. pyogenes* strains and the M serotyping.

References

Bert, F., Branger, C. & Lambert-Zechovsky, N. (1997) Pulsed-field gel electrophoresis is more discriminating than multilocus enzyme electrophoresis and random amplified polymorphic DNA analysis for typing pyrogenic streptococci. *Curr. Microbiol.*, **34**, 226-229.

Gardiner, D.L. & Sriprakash, K.S. (1996) Molecular epidemiology of impetiginous group A streptococcal infections in Aboriginal communities of Northern Australia. *J. Clin. Microbiol.*, **34**, 1448-1452.

Gruteke, P., Van Belkum, A., Schouls, L.M., Hendriks, W.D.H., Reubsaet, F.A.G., Dokter, J., Boxma, H. & Verbrugh, H.A. (1996) Outbreak of group A streptococci in a burn center: use of pheno- and genotypic procedures for strain tracking. *J. Clin. Microbiol.*, **34**, 114-118.

Gunzenhauser, J.D., Longfield, J.N., Brundage, J.F., Kaplan, E.L., Miller, R.N. & Brandt, C.A. (1995) Epidemic streptococcal disease among army trainees, July 1989 through June 1991. *J. Infect. Dis.*, **172**, 124-131.

Liu, P.Y.-F., Lau, Y.-J., Hu, B.-S., Shyr, J.-M., Shi, Z.-Y., Tsai, W.-S., Lin, Y.-H. & Tseng, C.-Y. (1995a) Analysis of clonal relationships among isolates of Shigella sonnei by different molecular typing methods. *J. Clin. Microbiol.*, **33**, 1779-1783.

Liu, P.Y.-F., Shi, Z.-Y., Lau, Y.-J., Hu, B.-S., Shyr, J.-M., Tsai, W.-S., Lin, Y.-H. & Tseng, C.-Y. (1995b) Comparison of different PCR approaches for characterization of *Burkholderia (Pseudomonas) cepacia* isolates. *J. Clin. Microbiol.*, **33**, 3304-3307.

Matsumoto, M., Miwa, Y., Matsui, H., Saito, M., Ohta, M. & Miyazaki, Y. (1999) An outbreak of pharyngitis caused by food-borne group A Streptococcus. *Jpn. J. Infect. Dis.*, **52**, 127-128.

Moody, M.D., Padula, J., Lizana, D. & Hall, C.T. (1965) Epidemiologic characterization of group A streptococci by T-agglutination and M-precipitation tests in public health laboratories. *Health Lab. Sci.*, **2**, 149-222.

Nakashima, K., Ichiyama, S., Iinuma, Y., Hasagawa, Y., Ohta, M., Ooe, K., Shimizu, Y., Igarashi, H., Murai, T. & Shimokata, K. (1997) A clinical and bacteriologic investigation of invasive streptococcal infections in Japan on the basis of serotypes, toxin production, and genomic DNA fingerprints. *Clin. Infect. Dis.*, **25**, 260-266.

Rotta, J., Krause, R.M., Lancefield, R.C., Everly, W. & Lackland, H. (1971) New approaches for the laboratory recognition of M types of group A streptococci. *J. Exp. Med.*, **134**, 1298-1315.

Struelens, M.J., Bax, R., Deplano, A., Quint, W.G. & VAN Belkum, A. (1993) Concordant clonal delineation of methicillin-resistant *Sta-*

phylococcus aureus by macrorestriction analysis and polymerase chain reaction genome fingerprinting. *J. Clin. Microbiol.*, **31**, 1964-1970.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H. & Swaminathan, B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.*, **33**, 2233-2239.

Yamamoto, T., Iyanaga, T. & Miyake, T. (1998) A mass outbreak of group A streptococcal pharyngitis among policemen in Fukuoka. [letter] *Intern. Med.*, **37**, 102.

Versalovic, J., Koeuth, T. & Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acid Res.*, **19**, 6823-6831.