17. Duncan, S., Barton, J. E. and O'Berin, P., *Mycol. Res.*, 1993, **97**, 1075–1082.
18. Liew, E. C. Y. and Irwin, J. A. G., *Mycol. Res.*, 1994, **98**, 1284–1290.
19. Wyss, P. and Bonfante, P., *Mycol. Res.*, 1993, **97**, 1351–1357.
20. Leach, J. E., White, F. M., Rhods, M. L. and Leung, H., *Mol. Plant–Microb. Interact*, 1990, **3**, 238–246.
21. Leach, J. E., Rhoads, M. L., VeraCruz, C. M., White, F. F., Mew, T. W. and Leung, H., *Appl. Environ. Microbiol.*, 1992, **58**, 2188–2195.
22. Adhikari, T. B., VeraCruz, C. M., Zhang, Q., Nelson, R. J., Skinner, D. Z., Mew, T. W. and Leach, J. E., *Appl. Environ. Microbiol.*, 1995, **61**, 966–971.
23. George, M. L. C., Leach, J. E. and Nelson, R. J., *Int. Rice Res. Inst. Newsl.*, 1995, 30–31.
24. Vera Cruz, C. M., Ardales, D. Z., Skinner, J. T., Nelson, R. J., Louws, F. J., Mew, T. W. and Leach, J. E., *Phytopathology*, 1996, **86**, 1352–1359.
25. Rajebhosale, M. D., Chowdhari, V. K., Ramakrishna, W., Tamhankar, S., Gnanamanickam, S. S., Gupta, V. S. and Ranjekar, P. K., *Theor. Appl. Genet.*, 1997, **95**, 103–111.
26. Chen, J., Lamikanra, O., Chang, C. J. and Hopkins, D. L., *Appl. Environ. Microbiol.*, 1995, **61**, 1688–1690.
27. Wetton, J. H., Carter Parkin, D. T. and Walters, D., *Nature*, 1987, **327**, 147–149.
28. Rhoads, D. D. and Roufa, D. J., SEQAID II, Kansas State Univ., Mol. Genet. Lab., Manhattan, 1989.
29. Reddy, A. P. K., in Proceedings of the International Workshop on Bacterial Blight of Rice, IRRI, 1989, pp. 79–88.
30. Rehman, F. U., Gnanamanaickam, S. S., Adhikari, T. B. and Alvarez, A. M., *Int. Rice. Res. Newsl.*, 1993, **18**, 33–34.
31. Swartz, D., *TAXAN 4.0 Users Manual*, Information Resource Group, Maryland Biotechnology Institute, University of Maryland, College Park, MD, USA.
32. Nelson, R. J., Baradian, M. R., Vera Cruz, C. M., Yap, I. M., Leach, J. E., Mew, T. and Leung, H., *Appl. Environ. Microbiol.*, 1994, **60**, 3275–3283.

ACKNOWLEDGEMENTS. We thank Dr A. P. K. Reddy, and Dr R. N. Misra for providing *X.o.* pv. *oryzae* isolates. M.D.R. is thankful to the Council of Scientific and Industrial Research, New Delhi for award of Senior Research Fellowship. This work was supported in part by the Rockefeller Foundation, USA. M.S. acknowledges the financial assistance and research facilities extended under Asian Rice Biotechnology Network.

Received 16 May 2000; revised accepted 19 January 2001

Application of PCR fingerprinting techniques for identification and discrimination of *Salmonella* isolates

Anjali Tikoo[†], A. K. Tripathi*, S. C. Verma*, N. Agrawal* and Gopal Nath[†,]**

[†]Department of Microbiology, Institute of Medical Sciences and *School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi 221 001, India

Different PCR-based DNA fingerprinting techniques were evaluated for identification and discrimination of bacterial strains. Fifteen strains of *Salmonella enterica* subspecies *enterica* serotypes Typhi (10), Paratyphi A (1) and Typhimurium (3) collected over a period of 15 years from stool, blood, and urine samples and river Ganga were tested. All the strains were analysed by restriction analysis of the amplified 16s rDNA (ARDRA), random amplified polymorphic DNA (RAPD) and BOX-PCR methods. In ARDRA, strains belonging to the same species were identified by identical fingerprints; RAPD on the other hand, divided *Salmonella* into 9 different groups. Two ciprofloxacin (CIP)-resistant strains showed similar PCR fingerprints which were different from the rest of the 12 strains. In BOX-PCR, all the strains of *Salmonella* showed 6 different groups which showed the presence of a common band. Serotype Typhimurium could be placed in the same group by BOX-PCR. CIP-resistant strains also produced one characteristic extra band by BOX-PCR. It was observed that RAPD had higher discriminatory power than BOX-PCR and was a simple and rapid technique for use in epidemiological studies of isolates belonging to *S. enterica*.

THE genus *Salmonella* consists of bacilli that are primarily parasites of the intestine of a large number of vertebrate species, including man. It causes enteric fever, gastroenteritis, septicaemia with or without focal suppuration and also carrier state in human beings. Epidemiological studies are done for the prevention and treatment of infections. The ability to distinguish isolates of *Salmonella* may be very important to trace the source of outbreak/s. The relationship, not only among the isolates from patients and environments, but also amongst the multiple isolations from the same patient may be interesting. The methods that have been used for deciphering the relatedness among the isolates are biotyping, antibiogram, plasmid typing, phage typing, serotyping, ribotyping and PCR fingerprinting. Some of the above techniques have low discriminatory potential, whereas others demand considerable amount of expertise, time and equipment.

**For correspondence. e-mail: gnath@banaras.ernet.in

Many reports are now available demonstrating the utility of PCR for typing of many organisms[1–3], including *Salmonella*. Utility of commonly used primers, targeted to REP (repetitive extragenic palindromic) and ERIC (enterobacterial repetitive intergenic consensus) sequence[4] elements in typing and differentiation of *Salmonella* isolates has been reported earlier. BOX-PCR assay, targeted to repetitive intergenic sequence elements of *Streptococcus*[5] and reported to produce species-specific and strain-specific genomic fingerprints, was used in the present study to investigate its utility for typing *Salmonella enterica* strains. The purpose of the present study was to evaluate the utility of PCR fingerprinting methods, viz. amplified ribosomal DNA restriction analysis (ARDRA), randomly amplified polymorphic DNA (RAPD) and BOX-PCR for identification and typing of different *Salmonella* isolates from various sources.

For the present study, 14 strains of *S. enterica* subspecies *enterica* serotypes Typhi (10), paratyphi A (1) and Typhimurium (3) and 1 strain of *Proteus mirabilis* isolated during the period 1983–1998, were subjected to PCR-based DNA fingerprinting. Of the 10 strains of serotype Typhi, 7 were isolated from blood, 2 from stool and 1 from urine samples. Of the 3 Typhimurium serotypes, 2 were isolated from stool samples and 1 from river Ganga. One strain of Paratyphi A was isolated from blood samples. All the isolates were isolated and identified by standard methods[6] and were maintained in the laboratory in peptone agar stab culture at room temperature. Table 1 shows the phage type and biotype of the 11 isolates.

The genomic DNA was isolated after growing cultures overnight at 37°C in Luria–Bertani (LB) medium, followed by lysis as described earlier[7]. Briefly, bacteria were suspended in a solution of 10 mM Tris HCl (pH 8.0), 1 mM EDTA, 1.0% SDS and proteinase K (100 mg/ml final concentration), and incubated for 60 min at 37°C. Then 5 M NaCl and 10% CTAB (hexadecyltrimethylammoniumbromide) were added and incubated for 10 min at 65°C. RNase was then added (approx. 30 μg/ml final concentration) and incubated for 30 min at 37°C. DNA was precipitated by adding an equal volume of isopropanol, followed by washing with 70% ethanol. The DNA was finally dissolved in 100 μl of sterile TE.

The amplification of 16s rDNA was performed in 50 μl reaction volume containing 5–10 ng template DNA, 5.0 μl 10X buffer, 1.5 mM $MgCl_2$, 1 μM forward primer (8f, 5'-AGAGTTTGATYMTGGCTCAG-3'), 1.0 μM reverse Primer (1492r, 5'-TACCTTGTTACGACTT-3'), 100 μM of each dNTP and 1 unit Taq DNA polymerase. The reaction mixture after incubation at 95°C for 8 min, was cycled 35 times through the following temperature profile: 94°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 1 min and finally it was incubated at 72°C for 5 min. A 4–6 μl aliquot of all the PCR-amplified 16s rDNA was used directly for restriction digestion with the restriction enzyme *Rsa*I. Restriction fragments were analysed by agarose gel (2.5%) electrophoresis.

In RAPD, each amplification reaction was performed in 40 μl final reaction mixture containing 10X buffer, 1 unit of Taq DNA polymerase, 4 μM of a 10-mer oligo primer (5'-AGCGCCATTG-3'), 200 μM dNTPs; 10–50 ng template DNA. PCR was performed with 35 cycles of 94°C for 1 min, 34°C for 2 min, 72°C for 1 min and 30 s and final extension at 72°C for 5 min and 60°C for 4 min. Amplified DNA products were resolved by electrophoresis on agarose 0.8% (W/V) gel. For BOX-PCR, a single primer corresponding to the BOX element[8] 5'-CATCGGCAAGGCGACGCTGACG-3' was

Table 1. Type, year, source, phage type and biotype of 14 different *Salmonella enterica* strains

Sl. no.	Strain no.	Type	Year	Source	Phage type	Biotype
1.	83/1	*Salmonella typhi*	1983	Stool sample	Vi neg.	I
2.	83/6	*S. typhi*	1983	Stool sample	Deg. Vi	I
3.	85/2	*S. typhimurium*	1985	Ganga water	UT	–
4.	86/8	*S. typhimurium*	1986	Stool culture	UT	–
5.	89/4	*S. typhimurium*	1989	Stool culture	UT	–
6.	89/7	*S. typhi*	1989	Stool culture	UVSI	I
7.	89/9	*S. typhi*	1989	Blood culture	ND	ND
8.	92/2	*S. typhi*	1992	Blood culture	A	I
9.	92/8	*S. typhi*	1992	Blood culture	EI	I
10.	92/24	*S. typhi*	1992	Blood culture	Vig neg.	I
11.	94/4	*S. typhi*	1994	Blood culture	40	I
12.	95/2	*S. typhi*	1995	Blood culture	EI	I
13.	–	Blank lane	–	–	–	–
14.	97/4	*Proteus mirabilis*	1988	Urine sample	–	–
15.	98/1	*S. typhi*	1998	Blood culture	ND	ND
16.	98/19	*S. paratyphi*	1998	Blood culture	ND	ND

ND, Not detected.

used. PCR cycling profiles were described by Selenska Pobell *et al.*[9] in a buffer consisting of 10 mM Tris HCl (pH 8.8), 50 mM KCl, 0.005% Tween 20, 0.01% gelatin, 1.5 mM $MgCl_2$ and 0.005% NP-40, 50 pmol of each primer and 2U of Taq polymerase in a 25 µl reaction mixture, with 50 ng of chromosomal DNA as template. Cycling conditions were as follows: 39 cycles at 95°C for 7 min, 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, with final extension at 72°C for 5 min and 60°C for 5 min. For analysis of amplification products, 15.0 µl of reaction mixture was used.

In ARDRA, when PCR amplicons were digested with *Rsa*I, 3 bands were observed (Figure 1). Excepting isolate 97/4, all other strains showed similar pattern with one additional band in strain 92/2. In view of these differences, phenotypic testing of strain 97/4 was carried out, which revealed that it was *Proteus mirabilis* and 92/2 was *Salmonella* serotype Typhi (*S. typhi*). However, RAPD fingerprinting of all the strains differentiated them in 9 groups (Figure 2). The two ciprofloxacin (CIP)-resistant isolates (83/6 and 89/9) were in the same group. Five strains, isolated during different years (83/1, 85/2, 86/8, 89/4 and 95/2) could be placed in one group. The strains 94/4 and 95/2 produced similar banding patterns with a difference of only one band. It may be noted that serotypes Typhi and Typhimurium shared many bands with each other, while Paratyphi-A (98/19) shared only a single common band with all the serotypes of *S. enterica*. Further, RAPD was able to differentiate strains having same phage types, i.e. Vi negative 83/1 and 92/24 and EI 92/8 and 95/2 (Table 1; Figure 2).

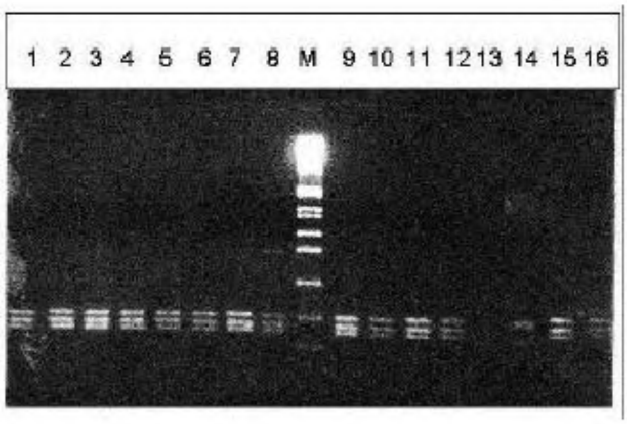

Figure 1. Amplified 16s rDNA of *Salmonella enterica* strains digested with *Rsa*I.

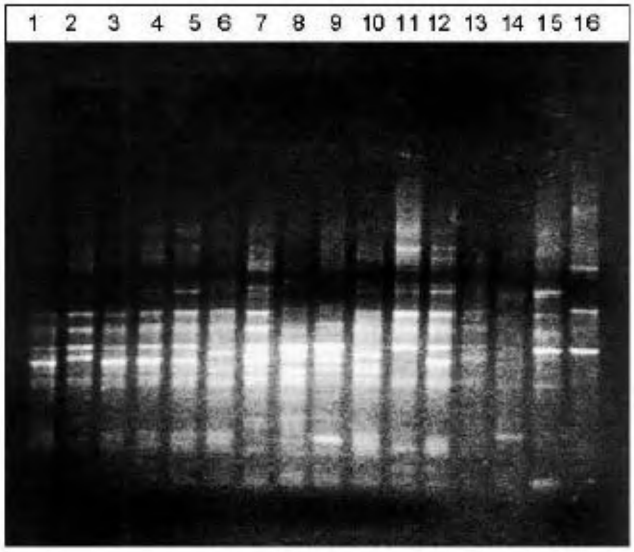

Figure 2. RAPD fingerprint of *Salmonella enterica* strains.

In BOX-PCR there were three common bands which were present in all the 14 strains. Another band which was amplified in 9 strains was absent in the rest of the 5 strains suggesting some degree of phylogenetic distance. The difference in the intensity of bands of the same size in different isolates results from point mutation, the template leading to reduced stability or poor annealing of the primer to these templates (Figure 3). A critical examination of the BOX-PCR results showed that 14 isolates could be placed in six groups. Eight of the various serotypes yielded a similar banding pattern, of which 3 were Typhimurium and 5 were Typhi serotypes. *S. paratyphi*-A (98/19) by BOX-PCR, again showed distinct banding pattern from the rest of the strains. Further, 2 of the CIP-resistant strains (83/6 and 89/9) produced similar banding pattern.

Several PCR fingerprinting methods have been used for typing clinical isolates of *Salmonella* and for studying their epidemiology. These techniques include RAPD, REP-PCR, ERIC-PCR and other variations using different oligo primers[10–12]. RAPD has been found to be the most powerful for discrimination among the strains. ERIC-PCR and REP-PCR have not been useful in differentiating *Salmonella* strains[11,12]. In the present study, we have used ARDRA, BOX-PCR and RAPD to

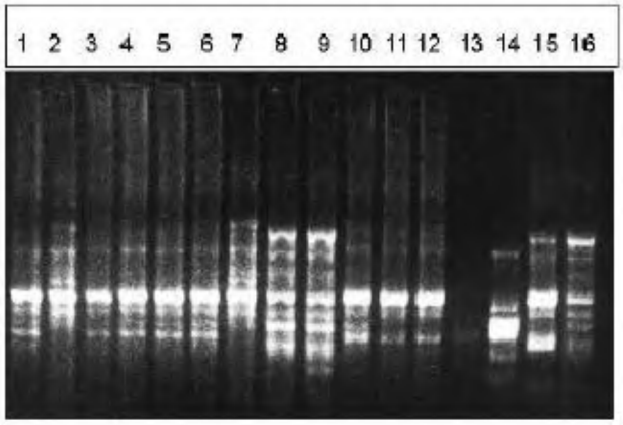

Figure 3. Fingerprint generated by BOX-PCR in *Salmonella enterica* strains.

differentiate the strains on the basis of their PCR fingerprints. Although RAPD has earlier been applied for the differentiation of *Salmonella* strains, BOX-PCR and ARDRA have not been applied so far for this purpose.

Ribotyping, which is based on the restriction fragment length polymorphism of the region encoding rRNAs, has earlier been used with some success for differentiation of *Salmonella* strains[12,13]. Notably, when we subjected PCR-amplified 16s rDNA to restriction digestion with *Rsa*I, no difference was observed in the restriction fragment pattern of all the 14 different serotypes of *S. enterica* subspecies *enterica*. The consistency and uniformity of the ARDRA pattern of all the 14 *Salmonella* strains, including serotypes Typhi, Paratyphi-A and Typhimurium, suggests that ARDRA could be used for rapid and definitive detection of *Salmonella*, which might be comparable to BAX system[14,15]. *P. mirabilis* (a member of family Enterobacteriaceae, like *Salmonella*) could be discriminated clearly (Figure 1). Similar banding pattern among all the serotypes approves the recent taxonomic status of the *Salmonella*[6]. However, an extra band in case of 92/2 might be due to restriction site modification in one of the several 16s rDNA of the rrn operons.

RAPD has proved to be the most convenient technique for discriminating intra-specific variations compared to many other PCR and non-PCR techniques like PT, RT and PFGE for *Salmonella*, as well as many other medically important bacteria[11,15–19]. It divided 14 strains of *Salmonella* into 9 groups, out of which the maximum number of strains, i.e. 5 were present in one group in the present study. Interestingly, the two CIP-resistant strains (83/6 and 89/9) could be put as one type due to the presence of an extra band. This extra band might be because of chromosomal location of the gene encoding ciprofloxacin resistance. We have observed that transformation of CIP-sensitive strains with plasmids isolated from CIP-resistant strains did not confer CIP resistance to the recipient. This suggested that genes encoding resistance to this drug might be located on the chromosome[20].

Three of the Typhimurium serotypes showed an identical pattern, out of which one was of environmental origin (river Ganga) and two clinical, indicating that all the 3 originated from the same clone. Surprisingly, all the 3 Typhimurium and 2 of Typhi serotypes produced similar banding pattern. This observation needs further study. However, difference in serotypes and virulence due to some extra chromosomal gene/s may be suggested. A single isolate of serotype Paratyphi-A had its distinct banding pattern. RAPD seems to be a very efficient discriminatory tool, because strains having the same phage type showed a different banding pattern.

BOX-PCR, using a primer with highly conserved repeated sequence which was longer than that used in RAPD, produced 6 patterns. BOX-PCR has earlier been reported to be useful for the discrimination of *Streptococcus pneumoniae*[5], Proteobacteria, e.g. *Xanthomonas*[8], *Burkholderia*[21] and *Rahnella*[9]. By this method strains belonging to serotype Typhimurium could be placed in the same group, whereas Paratyphi-A showed a distinct pattern. The 2 CIP-resistant strains again produced one extra band.

There are reports where RAPD results, after simple boiling of bacteria, have been found comparable with the results of alkaline lysis for DNA extraction, at least for *Salmonella*[18]. Our observations also indicated that RAPD was a convenient technique, which had higher discriminatory power than BOX-PCR. Therefore, RAPD may be considered as a rapid, highly discriminating and simple technique for typing of *Salmonella* isolates. The rapid and accurate identification and typing of *Salmonella* will be helpful in prevention, as well as cure of the diseases caused by it.

1. Manard, C., Brousseau, R. and Mouton. C., *FEMS Microbiol. Lett.*, 1992, **95**, 163–168.
2. Niesters, H. G. M., Goesseus, W. H. F., Meis, J. F. M. G. and Quint, W. G. V., *J. Clin. Microbiol.*, 1993, **31**, 904–910.
3. Struelens, H. J., Bax, R., Deplano, A., Quint, W. G. V. and Van Belkum, A., *J. Clin. Microbiol.*, 1993, **31**, 1964–1970.
4. Hulton, C. S. C., Higgins, C. F. and Sharp, P. M., *Mol. Microbiol.*, 1991, **5**, 825–834.
5. Martin, B. *et al.*, *Nucleic Acids Res.*, 1992, **20**, 3479–3483.
6. *Topley Wilson's Principles of Bacteriology, Virology and Immunity*, 1990, vol. II, 8th edn, pp. 469–493.
7. Bazzicalupo, M. and Fani, R., *Methods in Molecular Biology* (ed. Clap, J. P.), Humana Press Inc, Totowa, NJ, 1995, vol. 50, pp. 155–175.
8. Louws, F. J., Fulbright, D. W., Stephens, K. T. and de Bruijn, F., *Appl. Environ. Microbiol.*, 1994, **60**, 2286–2295.
9. Selenska Pobell, S., Evguenieva-Hackenberg, E. and Schwickerath, O., *Syst. Appl. Microbiol.*, 1995, **18**, 425–438.
10. Lopez Molina, N., Laconcha, I., Ramenteria, A., Audicana, A., Perales, I. and Garaizar, J., *J. Appl. Microbiol.*, 1998, **84**, 877–882.
11. Millemann, Y., Lesage Descauses, M. C., Lafont, J. P. and Chaslus Dancla, E., *FEMS Immunol. Med. Microbiol.*, 1996, **14**, 129–134.
12. Kerouanton, A., Brisabois, A., Grout, J. and Picard, B., *FEMS Immunol. Med. Microbiol.*, 1996, **14**, 25–29.
13. Landers, E., Gonzalez–Heria, M. A. and Mendoza, M. C., *Int. J. Food Microbiol.*, 1998, **43**, 81–90.
14. Bennett, A. R., Greenwood, D., Tennant, C., Banks, J. G. and Betts, R. P., *Lett. Appl. Microbiol.*, 1998, **26**, 437–441.
15. Hoorfer, J., Baggesen, D. L. and Porting, P. H., *J. Microbiol. Methods*, 1999, **35**, 77–84.
16. Lin, A. W., Usera, M. A., Barrett, T. J. and Goldsby, R. A., *J. Clin. Microbiol.*, 1996, **34**, 870–876.
17. Hitton, A. C., Banks, J. G. and Penn, C. W., *Lett. Appl. Microbiol.*, 1997, **24**, 243–248.
18. Kantama, L. and Jayanetra, P., *Southeast Asian J. Trop. Med. Public Health*, 1996, **27**, 119–125.
19. Shangkuan, Y. H. and Lin, H. C., *J. Appl. Microbiol.*, 1998, **85**, 693–702.
20. Lewin, C. S., *J. Med. Microbiol.*, 1992, **36**, 9–11.
21. Smith, J. J., Ollord, L. C., Holderness, M. and Saddler, G. S., *Appl. Environ. Microbiol.*, 1995, **16**, 4263–4268.

Received 25 September 2000; revised accepted 10 January 2001