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rDNA fingerprinting as a tool in epidemiological analysis of *Salmonella typhi* infections

A. NASTASI, C. MAMMINA AND M. R. VILLAFRATE

Department of Hygiene & Microbiology 'G. D'Alessandro', Center for Enterobacteriaceae of Southern Italy, University of Palermo, via del Vespro 133, 90127 Palermo, Italy

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SUMMARY

Characterization of 169 strains of *Salmonella typhi* of phage types C_1, C_4, D_1 and D_9 isolated in 1975–88 was carried out by rDNA gene restriction pattern analysis. Twenty-four isolates had been recovered during four large waterbone outbreaks in the last 20 years in Sicily; 145 strains, isolated from apparently sporadic cases of infection in Southern Italy in the same period of time, were also examined.

Application of rRNA–DNA hybridization technique after digestion of chromosomal DNA with *Cla* I showed the identity of patterns of the epidemic strains of phage types C_1 and D_1, confirming attribution of the outbreaks to single bacterial clones. Patterns of the two available strains of lysotype D_9 were slightly different, whilst the 12 epidemic strains of phage type C_4 could be assigned to two distinct patterns scarcely related to each other and, consequently, to two different clones. A considerable heterogeneity was detected among all apparently sporadic isolates of the four phage types under study.

This fingerprinting method appears a reliable tool to complement phage typing in characterizing isolates of *S. typhi*. In particular, epidemiological features of spread of this salmonella serovar in areas, where simultaneous circulation of indigenous and imported strains occurs, can be elucidated.

INTRODUCTION

Typhoid fever is still a serious public health problem in many geographic areas. Large outbreaks frequently involve developing regions, but epidemics have also been reported in recent years from developed countries [1].

Characterization of *Salmonella typhi* isolates traditionally depends on bacteriophage typing, this serovar being very homogeneous with respect to other phenotypic characteristics, such as biochemical reactions and susceptibility to antimicrobial drugs. However, phage typing often fails to provide useful epidemiological information, because of the frequent occurrence of some certain phage types [2, 3]. Furthermore, possible changes of lysis pattern may occur as a result of acquisition or loss of lysogenic phages [4].

Molecular techniques have recently been applied to investigations on epidemic and endemic strains of *S. typhi*. Restriction endonuclease digestion pattern

analysis of chromosomal DNA and multilocus enzyme electrophoresis have shown a high degree of homogeneity among *S. typhi* isolates [5–7], whereas rDNA gene restriction pattern analysis [8, 9] has identified remarkable genomic variations among strains and thus appeared to be a reliable and sensitive technique, able to complement phage typing in further differentiation of strains [10].

In the last 20 years four large outbreaks of typhoid fever occurred in Sicily in 1977, 1980, 1984 and 1988, which were linked to phage types D_9, C_4, C_1 and D_1, respectively. In this period C_4, C_1 and D_1 were, after lysotype A, the most frequently identified phage types in Southern Italy, the D_1 being the commonest type in Sicily [11].

The aim of this study was to elucidate epidemiological features of the spread in our geographic area of epidemic and endemic strains of *S. typhi* of the lysotypes C_1, C_4, D_1 and D_9 by the application of rDNA gene restriction pattern analysis.

MATERIALS AND METHODS

Bacterial strains

One hundred and sixty-nine strains of *S. typhi* of the phage types C_1, C_4, D_1 and D_9 were selected from the culture collection of the Southern Italy Centre of Enterobacteriaceae of Palermo. Twenty-four isolates had been recovered from patients and healthy carriers during investigation of four large typhoid fever outbreaks, caused by these phage types in the years 1975–88 in Sicily; 145 strains of the same phage types were also included, that had been isolated in Southern Italy in the same period of time from apparently sporadic cases of infection (Table 1). In particular, approximately 50% of available strains isolated in the years 1975–88 for the phage type C_4 and in the years 1980–88 for the lysotypes C_1 and D_1 were selected; moreover, all available strains of the phage type D_9 isolated between 1975 and 1988 were analysed, because of infrequent identification of this lysotype.

Isolates were phage typed by the Vi phage typing scheme of Anderson and Williams [12].

Isolation of DNA

For whole-cell DNA preparation, organisms from overnight 10 ml broth cultures were harvested, washed in saline and resuspended in 400 μl of 150 mM NaCl, 10 mM Tris-HCl (pH 8) and 10 mM EDTA. RNAse (20 μl of 5 mg/ml) and proteinase K (60 μl of 0·5 mg/ml) were added; then, bacterial cells were treated with 20 μl of 10% (w/v in water) sodium dodecyl sulphate and incubated in a water bath at 50 °C for 30 min. Thereafter, DNA was submitted to several extractions with phenol-chloroform-isoamylalcohol (25:24:1) and chloroform-isoamylalcohol (24:1) and ethanol precipitated at minus 20 °C. The pellet was resuspended in water and the concentration and purity of DNA were evaluated by agarose gel electrophoresis.

Photobiotin labelling of rRNA

Commercially available rRNA from *Escherichia coli* (Sigma) was labelled by the technique described by Forster and co-workers [13]. Briefly, equal volumes of the

Table 1. *Strains of* Salmonella typhi *isolated in Southern Italy in the years 1975–88*

Phage type	No. of strains	Place and time of isolation			*Cla* I ribotype
D9	1	Palermo	Sicily	1975	4
	2	Catanzaro	Calabria	1976	1
	1*	Caltanissetta	Sicily	1977	1
	1*	Caltanissetta	Sicily	1977	2
	1	Catanzaro	Calabria	1979	1
	1	Palermo	Sicily	1980	4
	1	Palermo	Sicily	1982	4
	1	Naples	Campania	1983	5
	1	Palermo	Sicily	1984	4
	1	Palermo	Sicily	1985	4
	1	Palermo	Sicily	1985	6
	1	Palermo	Sicily	1985	7
	1	Brindisi	Apulia	1986	9
	1	Palermo	Sicily	1986	3
	1	Palermo	Sicily	1986	4
	1	Palermo	Sicily	1986	8
C4	1	Catania	Sicily	1975	12
	1	Catania	Sicily	1975	24
	1	Catanzaro	Calabria	1975	13
	1	Catanzaro	Calabria	1975	11
	1	Naples	Campania	1975	10
	1	Catanzaro	Calabria	1980	10
	7*	Enna	Sicily	1980	10
	5*	Enna	Sicily	1980	11
	1	Caserta	Campania	1982	16
	1	Catania	Sicily	1982	14
	1	Catania	Sicily	1982	15
	1	Messina	Sicily	1982	10
	1	Avellino	Campania	1983	11
	1	Catanzaro	Calabria	1983	10
	1	Naples	Campania	1983	17
	1	Potenza	Basilicata	1984	18
	2	Catania	Sicily	1985	10
	2	Catania	Sicily	1985	19
	2	Brindisi	Apulia	1986	10
	3	Brindisi	Apulia	1987	10
	1	Brindisi	Apulia	1988	10
	3	Brindisi	Apulia	1988	20
	3	Brindisi	Apulia	1988	21
	1	Brindisi	Apulia	1988	22
	1	Catania	Sicily	1988	17
	1	Catania	Sicily	1988	23
C1	1	Catania	Sicily	1980	29
	1	Catania	Sicily	1980	4
	1	Catania	Sicily	1980	37
	2	Catanzaro	Calabria	1980	28
	1	Reggio C.	Calabria	1980	36
	1	Avellino	Campania	1981	30
	1	Caltanissetta	Sicily	1982	26
	1	Enna	Sicily	1982	31
	1	Palermo	Sicily	1982	25
	1	Palermo	Sicily	1982	27

Phage type	No. of strains	Place and time of isolation			*Cla* I ribotype
	1	Palermo	Sicily	1982	28
	1	Potenza	Basilicata	1982	30
	1	Reggio C.	Calabria	1982	27
	2	Palermo	Sicily	1983	27
	5*	Agrigento	Sicily	1984	25
	5	Caltanissetta	Sicily	1984	26
	1	Catanzaro	Sicily	1984	28
	1	Messina	Sicily	1984	27
	1	Palermo	Sicily	1984	25
	1	Palermo	Sicily	1984	27
	1	Messina	Sicily	1985	4
	2	Brindisi	Apulia	1986	28
	1	Brindisi	Apulia	1986	29
	1	Brindisi	Apulia	1986	34
	1	Catania	Sicily	1986	27
	1	Catania	Sicily	1986	30
	1	Catania	Sicily	1986	4
	1	Palermo	Sicily	1986	28
	2	Brindisi	Apulia	1987	28
	1	Brindisi	Apulia	1988	35
	1	Brindisi	Apulia	1988	28
	1	Caserta	Campania	1988	29
	1	Caserta	Campania	1988	30
	1	Enna	Sicily	1988	32
	1	Brindisi	Apulia	1988	27
	1	Brindisi	Apulia	1988	28
	1	Brindisi	Apulia	1988	33
	1	Brindisi	Apulia	1988	19
D1	1	Cantania	Sicily	1980	51
	1	Catanzaro	Calabria	1980	54
	1	Catanzaro	Calabria	1980	58
	1	Palermo	Sicily	1980	4
	2	Palermo	Sicily	1980	53
	1	Palermo	Sicily	1980	55
	1	Palermo	Sicily	1980	56
	1	Palermo	Sicily	1980	57
	2	Palermo	Sicily	1980	60
	1	Siracusa	Sicily	1980	38
	1	Siracusa	Sicily	1980	52
	1	Palermo	Sicily	1981	38
	1	Palermo	Sicily	1981	39
	1	Palermo	Sicily	1981	40
	1	Palermo	Sicily	1981	59
	1	Palermo	Sicily	1981	60
	1	Palermo	Sicily	1981	61
	1	Palermo	Sicily	1982	62
	1	Palermo	Sicily	1982	63
	1	Naples	Campania	1983	4
	1	Palermo	Sicily	1983	4
	1	Palermo	Sicily	1983	38
	1	Palermo	Sicily	1983	40
	3	Palermo	Sicily	1983	41
	1	Palermo	Sicily	1983	42
	1	Palermo	Sicily	1983	43
	1	Palermo	Sicily	1983	44

Phage type	No. of strains	Place and time of isolation			*Cla* I ribotype
	1	Siracusa	Sicily	1983	4
	1	Catanzaro	Calabria	1984	50
	1	Messina	Sicily	1984	45
	1	Palermo	Sicily	1984	45
	1	Palermo	Sicily	1984	49
	1	Palermo	Sicily	1985	48
	1	Brindisi	Apulia	1986	4
	1	Brindisi	Apulia	1986	47
	1	Catania	Sicily	1986	46
	1	Palermo	Sicily	1986	19
	2	Palermo	Sicily	1986	38
	5	Brindisi	Apulia	1987	4
	1	Palermo	Sicily	1987	38
	1	Palermo	Sicily	1988	4
	1	Palermo	Sicily	1988	39
	1	Palermo	Sicily	1988	40
	5*	Siracusa	Sicily	1988	38

* Epidemic strains.

rRNA sample (1 μg/μl) and of a photobiotin acetate (Sigma) solution (1 μg/μl) were mixed in a sterile microcentrifuge tube and irradiated in an ice bath, 10 cm below a 250 Watt sunlamp for 20 min. Thereafter, excess photolysed photobiotin was removed by repeated 2-butanol extractions. The biotin labelled rRNA was ethanol precipitated overnight at -20 °C. Then the pellet was resuspended in 0·1 mM Na_2EDTA. The probe concentration was determined by measuring the absorbance at 260 nm. Phage lambda DNA was photobiotin labelled by the same procedure.

rDNA gene restriction pattern analysis

Approximately 2 μg of bacterial DNA were digested with restriction endonucleases (Gibco-BRL Ltd) under conditions recommended by the supplier. Photobiotin labelled lambda DNA was also digested by *Hind* III to provide molecular weight markers. Resulting fragments were separated by overnight electrophoresis on 0·8% w/v agarose (Gibco-BRL Ltd) in Tris-borate buffer (0·089 M Tris-borate, 0·002 M EDTA) as previously described [14].

DNA was partially depurinated in 0·25 N HCl for 5 min, then denatured in 1·5 M NaCl-0·5 M NaOH for 30 min and neutralized in 1·5 M NaCl–0·5 M Tris-HCl, pH 7·5 for 30 min. DNA was transferred to nitrocellulose filters by vacuum-assisted transfer. After completion of the transfer, nitrocellulose membranes were washed once in 2X SSC (1X SSC is 0·15 M NaCl–0·015 M trisodium citrate, pH 7·0), air dried, baked at 80 °C for 1·5 h and finally stored at 4 °C.

Nitrocellulose filters were prehybridized at 42 °C for 6–8 h in a solution containing 50% v/v formamide, 5X SSC, 5X Denhardt's solution (1X Denhardt's solution is 0·02% polyvinylpyrrolidone, 0·02% Ficoll 400, 0·02% bovine serum albumin), 0·5 M sodium phosphate pH 6·8, 1% w/v SDS and 100 μg/ml of freshly denatured sheared salmon sperm DNA and yeast tRNA. The membranes were

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hybridized with the photobiotin labelled rRNA (250 ng/ml of hybridization solution) at 42 °C overnight in a solution containing 50% formamide, 5X SSC, 1X Denhardt's solution, 0·2 M sodium phosphate pH 6·8, 2% SDS and 100 μg/ml of salmon sperm DNA and yeast tRNA. After hybridization, nitrocellulose filters were washed twice at room temperature for 5 min in 0·1% SDS-2X SSC, twice at 60 °C for 1 h in 0·1% SDS-2X SSC and once at room temperature in 2X SSC. The hybridized bands were visualized by the 'BLUGENE' non-radioactive nucleic acid detection system (Gibco-BRL Ltd) as suggested by the manufacturer.

Statistical methods

Cla I rDNA patterns within each lysotype were screened for the total of bands and presence or absence was coded as 1 and 0, respectively. The similarity coefficient between each pair of ribotypes was expressed as the fraction of zero and non-zero matches to the total number of hybridized bands used in the comparison. Clustering was carried out from a matrix of coefficients of pairwise genetic distances using the commercial program STATPRO (Penton Software, Inc.).

RESULTS

Several restriction endonucleases were tested on a sample of *S. typhi* isolates to evaluate their suitability in differentiation of strains; *Hinc* II and *Cla* I were selected.

Cleavage with *Hinc* II yielded 12 distinct patterns among the 169 isolates under study (Fig. 1). Lysotype C_1 showed more variability among strains than the other phage types: the epidemic strains and 14 of the 45 non-epidemic isolates belonged to ribotype 1, whereas among the remaining 31 isolates *Hinc* II ribotypes 2 to 10 were identified. All isolates of phage type D_1 and all but one of phage type D_9 were attributed to the ribotype 1. In contrast, the epidemic strains and the majority (23 of 33) of the non epidemic isolates of phage type C_4 belonged to the ribotype 2.

The best discrimination among strains was obtained by cleavage of chromosomal DNA with *Cla* I, which generated large restriction fragments. Sixty-three different rDNA hybridization patterns were observed, of which only two, 4 and 19, were shared by different phage types. Figure 2 shows the rRNA-DNA hybridization profiles of epidemic strains and the most frequent ribotypes of non epidemic strains within each lysotype. The results of rDNA fingerprinting of the *S. typhi* strains under study could be summarized as follows.

Phage type D_9

Until 1970 this phage type was considered to be characteristic of the Middle East area; it was first identified in Naples during 1973 and in Sicily in 1975 [11]. During summer 1977 it caused a large waterborne outbreak in Caltanissetta (Sicily). Only two strains from this event were available, which after rRNA hybridization of their *Cla* I digested chromosomal DNA showed two slightly different rDNA fingerprints – 1 and 2 (Figs. 2 and 3). A considerable genetic heterogeneity was detected, despite their small number, among all isolates of lysotype D_9 identified during the years 1975–88.

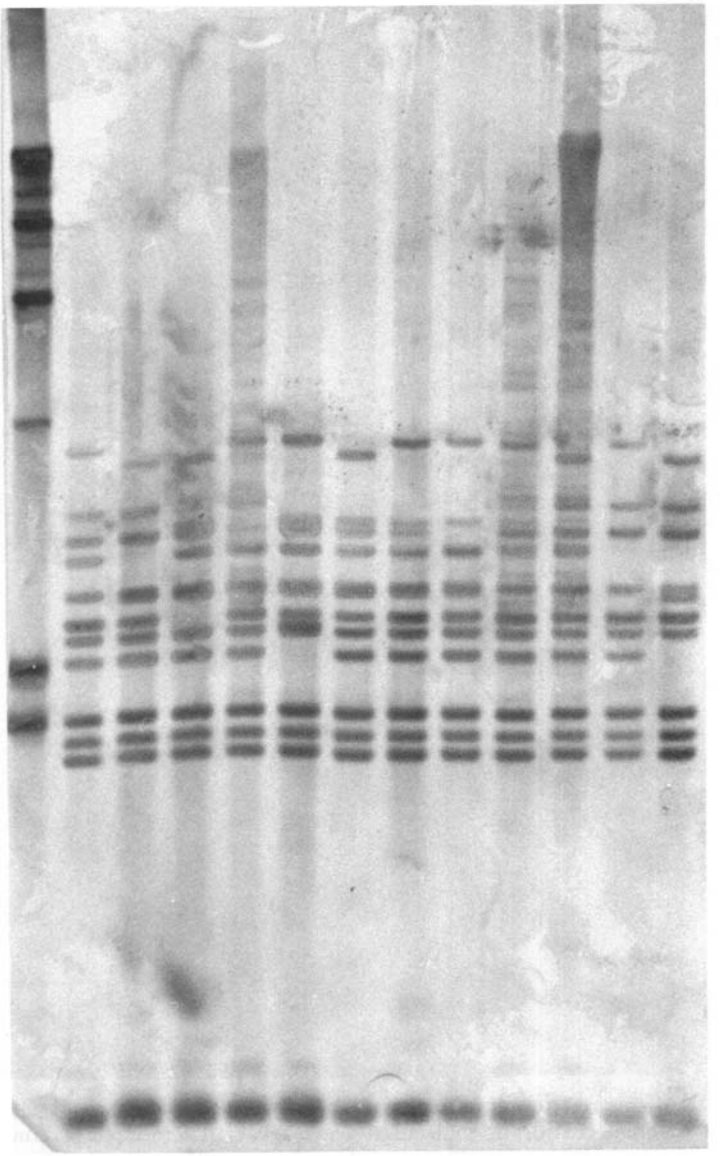

Fig. 1. rDNA gene restriction patterns of *Hinc* II digests of strains of *S. typhi*. Lanes: 1. molecular size markers (bacteriophage lambda DNA cleaved with *Hind* III; 2 to 13. ribotypes 1 to 12.

Phage type C_4

This is a predominant phage type in Southern Italy [11]. In November–December 1980 it was responsible of a waterborne outbreak which caused approximately 70 cases in Piazza Armerina (Enna, Sicily). Two clearly different rRNA hybridization patterns, 10 and 11, (Figs 2 and 3) were identified after *Cla* I digestion of chromosomal DNA of five epidemic strains. Analysis of seven additional isolates from the same outbreak confirmed this finding. rRNA

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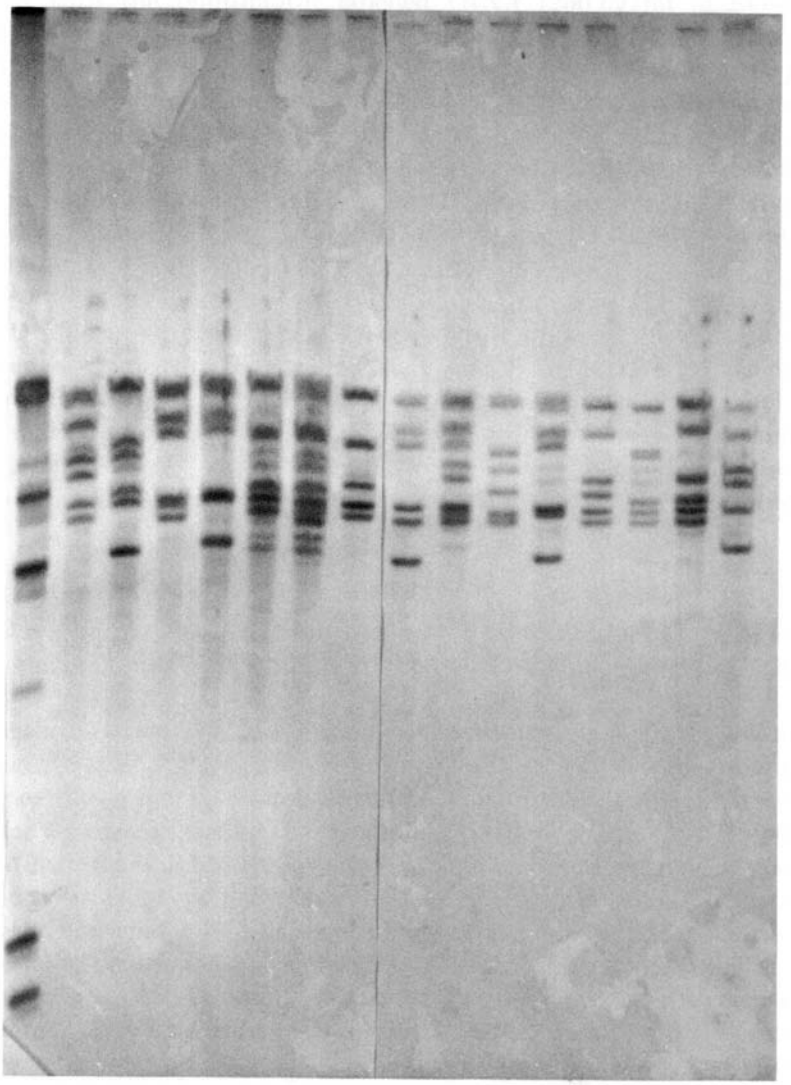

Fig. 2. rDNA gene restriction patterns of *Cla* I digests of epidemic and non epidemic strains of *S. typhi*. Lanes: 1, molecular size markers (bacteriophage lambda DNA cleaved with *Hind* III; 2 to 5, phage type C_1: ribotypes 25 (epidemic), 26, 27 and 28; 6 to 8, phage type D_9: ribotypes 1 (epidemic), 2 (epidemic) and 5; 9 to 12, phage type C_4: ribotypes 10 (epidemic), 11 (epidemic), 20 and 21; 13 to 16: phage type D_1: ribotypes 38 (epidemic), 4, 40 and 41.

hybridization of chromosomal DNA cleaved by *Bgl* II (data not shown) and *Eco*R I (Fig. 4) also assigned seven and five epidemic isolates, respectively, to two distinct profiles. High variability was detected among non epidemic strains isolated between 1975 and 1988: 15 ribotypes were observed among 33 isolates by *Cla* I digestion. The epidemic pattern 10 was also the most frequently identified among the apparently sporadic isolates. Ten strains isolated in Brindisi in the period 1986–8 were characterized by the related ribotypes 10, 21 and 22 (Fig. 3).

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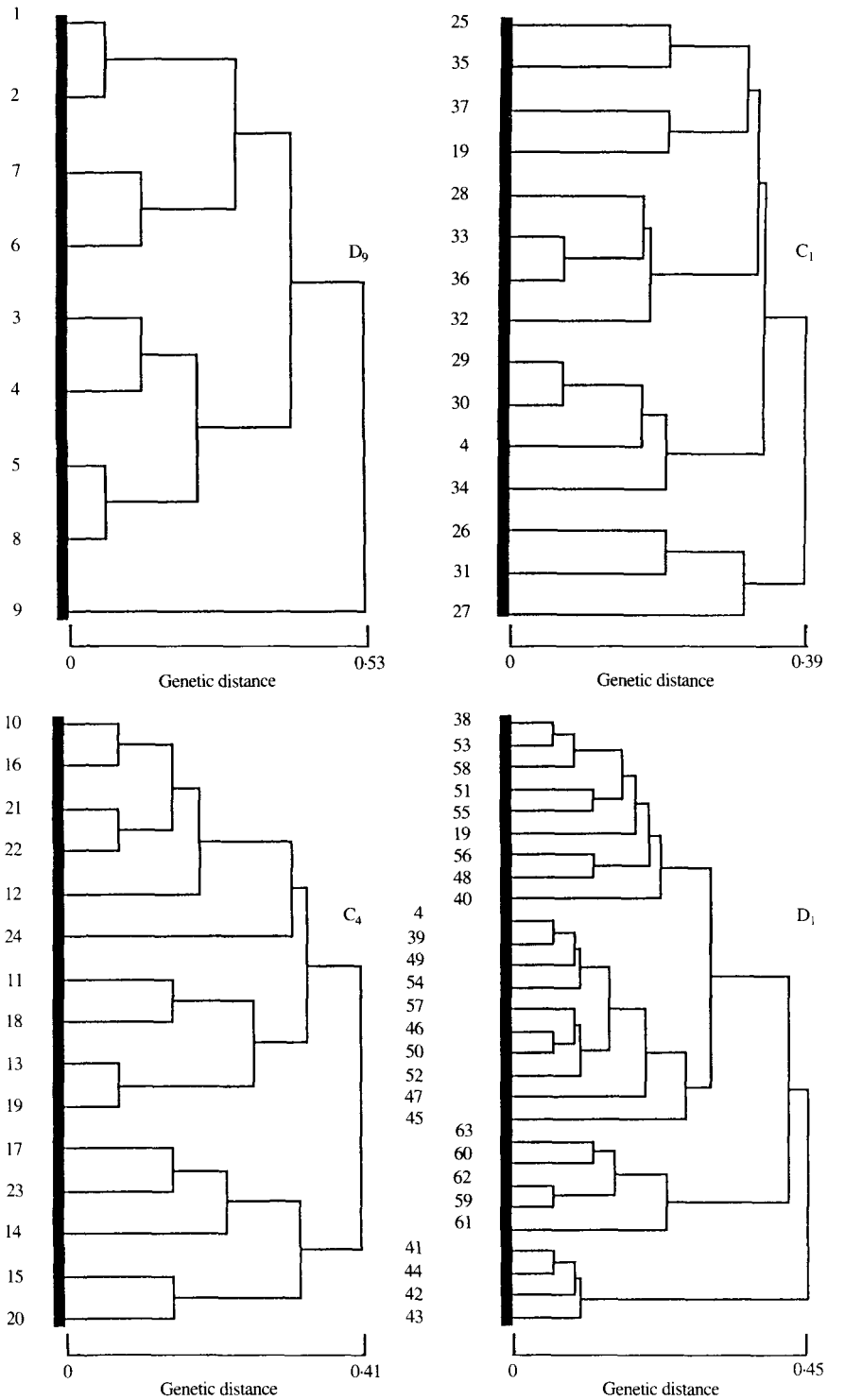

Fig. 3. Clustering of *Cla* I rDNA gene restriction patterns of *S. typhi* strains of lysotipes C_1, C_4, D_1 and D_9. Ribotypes numbers refer to those of Table.

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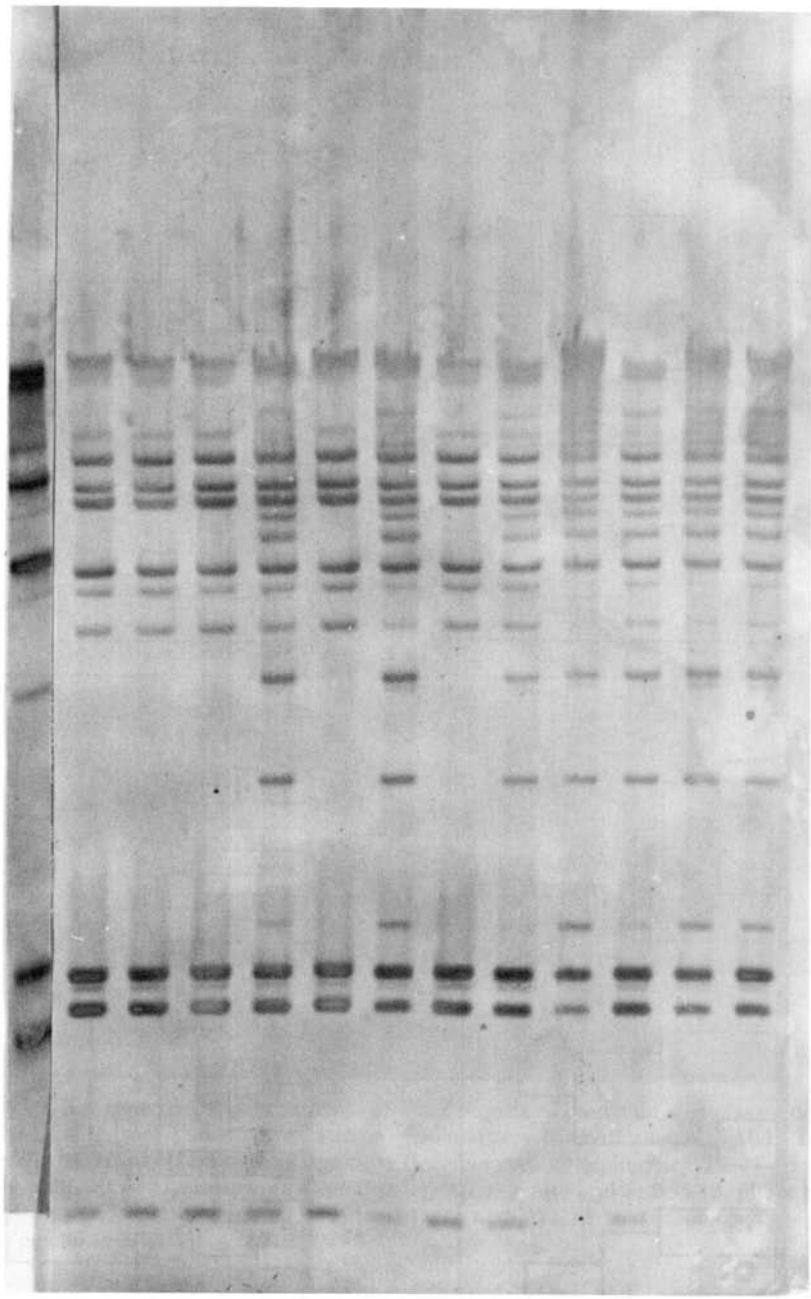

Fig. 4. rDNA gene restriction patterns of *Eco*R I digests of epidemic strains of *S. typhi* of phage type C_4. In lane 1. molecular size markers (bacteriophage lambda DNA cleaved with *Hin*d III).

Phage type C_1

Since 1980 this cosmopolitan phage type has been increasingly identified in Southern Italy. In the period August–September 1984 a large waterborne outbreak of at least 100 cases occurred in Calamonaci (Agrigento, Sicily). Five

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S. typhi strains isolated during this epidemic exhibited indistinguishable rRNA gene restriction patterns, ribotype 25, after *Cla* I digestion. rDNA fingerprints of non-epidemic isolates were polymorphic. Pattern 26 was uniquely found in five strains from apparently sporadic cases of disease occurred in Gela (Caltanissetta, Sicily) during September 1984 and in one isolate recovered from the same town in 1982. This latter ribotype was scarcely related to the epidemic pattern 25 (Fig. 3).

Phage type D_1

This phage type is the most frequently encountered in Sicily [11]. In December 1988 it caused a waterborne outbreak in Avola (Siracusa, Sicily). Five epidemic isolates were submitted to rRNA gene restriction pattern analysis and had an identical pattern, ribotype 38. This pattern was given also by one strain of *S. typhi* previously isolated from the same geographic area. There were 28 ribotypes identified among the 52 non-epidemic isolates examined. Five apparently sporadic isolates recovered in Brindisi (Apulia) during 1987 shared the same ribotype 4. Furthermore the cluster containing the ribotypes 59, 60, 61, 62 and 63 and the cluster containing the ribotypes 41, 42, 43 and 44 seemed to be exclusively related to isolates identified in Palermo in the years 1983 and 1981–2.

DISCUSSION

Application of the rRNA–DNA hybridization technique on epidemic and non-epidemic strains of *S. typhi* provided useful data towards epidemiological analysis of spread of some phage types in Southern Italy. The results of molecular typing by rDNA pattern analysis of *Cla* I digested chromosomal DNA suggests the following considerations.

(a) Two of the waterborne typhoid outbreaks, in particular those of Calamonaci in 1984 and of Avola in 1988 due to phage types C_1 and D_1, respectively, may be attributed to a single bacterial clone. In the epidemic caused by the phage type D_9 in 1977 the two strains examined exhibited slightly different patterns, that because of their high degree of similarity may be presumably considered as expression of spread of a single clone of *S. typhi*. Minor differences among epidemiologically related strains might be perhaps expected since point mutations may affect, though rarely, restriction endonuclease sites. Unfortunately, additional strains from this outbreak were unavailable for molecular analysis in order to test these hypotheses. In contrast, the epidemic isolates of phage type C4 were undoubtedly subdivided into two distinct clones, since differences obtained with *Cla* I cleavage of chromosomal DNA were confirmed by the use of *Bgl* II and *Eco*R I. Consequently, simultaneous involvement in the outbreak of two different clones of *S. typhi* of phage type C_4 must be presumed.

(b) A remarkable heterogeneity of rDNA gene restriction patterns was observed among non-epidemic strains of all phage types under study. A similar finding characterized also the phage type D_9 despite of its infrequent isolation and apparently recent emergence in Southern Italy. Thus a notable number of bacterial clones seems to support the endemic circulation of the four lysotypes examined in this geographic area.

Strain characterization by rDNA gene restriction pattern analysis in association

with phage typing may effectively contribute to identification and investigation of outbreaks and to trace epidemiologically related and unrelated isolates of *S. typhi*. This technique appears of particular interest in geographic areas, such as Southern Italy, where importation of strains by the increasing immigration from developing countries is associated to the endemic persistence of indigenous strains.

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