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Molecular relationship among *Salmonella dublin* isolates identified at the Center for Enterobacteriaceae of Palermo during the years 1971–85

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SUMMARY

A molecular epidemiological study was carried out on 60 *Salmonella dublin* isolates identified at the Southern Italy Enterobacteriaceae Center between 1971 and 1985. These included 23 isolates from children with diarrhoea in Palermo obtained during 1984.

All isolates from the outbreak of gastroenteritis in children were resistant to chloramphenicol and streptomycin and harboured two plasmids of 50 MDa and 3 MDa molecular weight, whereas the majority of the isolates identified before 1984 were susceptible to these antibiotics and carried only a 50 MDa molecular weight plasmid. Four *S. dublin* strains successively identified from cattle (Palermo, Foggia, Portici) and from a child (Palermo) were shown to possess similar antibiotic resistance patterns and plasmid profiles to *S. dublin* isolates from the outbreak of gastroenteritis in children.

The 50 MDa plasmid was shown to be associated with virulence in mice, while it was not possible to assign any genetic function to the 3 MDa plasmid.

INTRODUCTION

In recent years it has been shown that plasmids play an important role in mediating virulence properties of salmonella serotypes such as *Salmonella typhimurium* (Jones *et al.* 1982; Baird, Manning & Jones, 1985; Helmuth *et al.* 1985), *S. enteritidis* (Helmuth *et al.* 1985; Nakamura *et al.* 1985) and *S. choleraesuis* (Helmuth *et al.* 1985). In *S. dublin*, a serotype which causes invasive disease in cattle and humans, a 50 MDa plasmid has been reported to be associated with the virulence for mice (Terakado *et al.* 1983; Baird, Manning & Jones, 1985; Chikami, Fierer & Guiney, 1985; Manning, Baird & Jones, 1986). Furthermore, a high degree of DNA-sequence homology has been found between the virulence-associated plasmids of *S. dublin* and *S. typhimurium* (Baird, Manning & Jones, 1985) and between these plasmids and those of other salmonella serotypes (Popoff *et al.* 1984). In Southern Italy *S. dublin* was usually isolated from cattle until 1984, when it was associated with an outbreak of gastroenteritis in children in Palermo.

Table 1. Salmonella dublin *isolate identified at CEPIM during the years 1971–85*

Year of isolation	Strain	Source	Place of isolation	Antibiotic* resistance	MWs of plasmids identified (MDa)
1971	7229	Human	Palermo	—	50 — —
	7232		Palermo	—	50 25 —
	7621	Cattle	Palermo	—	— ——
	7636		Palermo	—	50 — —
	7645		Palermo	—	50 — —
	7647	Human	Palermo	—	50 — —
	7655	Cattle	Palermo	—	— — —
	7658	Human	Palermo	—	50 — —
	7666	Cattle	Palermo	—	50 — —
	7669	Human	Palermo	—	50 — —
	7675		Palermo	—	50 — —
	7678	Cattle	Palermo	—	50 — —
	7682	Human	Palermo	—	50 — —
1973	8656	Cattle	Palermo	—	50 — —
	8722		Portici	—	— — —
	8729		Portici	—	50 — —
1975	10739		Portici	Tc	50 — 3·0
	10868		Portici	Cm Sm	50 — —
1976	11478		Palermo	Cm Sm	50 — 3·0
	11652		Palermo	—	50 — —
1977	13144		Palermo	Cm Sm	50 — —
1980	15007	Human	Palermo	Ap Cm Sm Tc	50 — —
	15586	Cattle	Palermo	—	50 — —
1981	16813		Palermo	—	50 — —
	16814		Palermo	—	50 — —
	17263	Human	Caserta	—	— — —
	17605	Cattle	Messina	—	50 — 3·0
1982	19878	Human	Palermo	—	50 — 3·0
	20572		Brindisi	—	50 — —
1983	20991	Cattle	Palermo	—	50 — —
	20996		Palermo	—	50 — —
	20997		Palermo	—	50 — —
1984	23078	Cattle	Foggia	—	50 — —
	23473	Human	Palermo	Cm Sm	50 — 3·0
	23475		Palermo	Cm Sm	50 — 3·0
	23476		Palermo	Cm Sm	50 — 3·0
	23477		Palermo	Cm Sm	50 — 3·0
	23478		Palermo	Cm Sm	50 — 3·0
	23480		Palermo	Cm Sm	50 — 3·0
	23481		Palermo	Cm Sm	50 — 3·0
	23482		Palermo	Cm Sm	50 — 3·0
	23483		Palermo	Cm Sm	50 — 3·0
	23486		Palermo	Cm Sm	50 — 3·0
	23487		Palermo	Cm Sm	50 — 3·0
	23488		Palermo	Cm Sm	50 — 3·0
	23489		Palermo	Cm Sm	50 — 3·0
	23490		Palermo	Cm Sm	50 — 3·0
	23493		Palermo	Cm Sm	50 — 3·0
	23494		Palermo	Cm Sm	50 — 3·0
	23495		Palermo	Cm Sm	50 — 3·0
	23496		Palermo	Cm Sm	50 — 3·0
	23511		Palermo	Cm Sm	50 — 3·0
	23537		Palermo	Cm Sm	50 — 3·0
	23562		Palermo	Cm Sm	50 — 3·0

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Table 1. (*cont*)

Year of isolation	Strain	Source	Place of isolation	Antibiotic* resistance	MWs of plasmids identified (MDa)
	23625	Cattle	Palermo	Cm Sm	50 — 3·0
	23790	Human	Palermo	Cm Sm	50 — 3·0
	23854		Palermo	Cm Sm	50 — 3·0
1985	24329	Cattle	Foggia	Cm Sm	50 — 3·0
	24516		Portici	Cm Sm	50 — 3·0
	24949	Human	Palermo	Cm Sm	50 — 3·0

* Ap, Ampicillin; Cm, Chloramphenicol; Sm, Streptomycin; Tc, Tetracycline.

This paper describes an investigation in which the antibiotic resistance patterns and plasmid DNA profiles of the isolates from the children are compared with other *S. dublin* isolates identified at the Southern Italy Enterobacteriaceae Center during the period 1971–85.

MATERIALS AND METHODS

Bacterial strains

Sixty *S. dublin* isolates sent for identification to the Southern Italy Enterobacteriaceae Center (CEPIM) during the years 1971–85 were studied (Table 1). Twenty-five of these were obtained during 1984 and 23 were from a diarrhoeal disease outbreak in children which occurred in Palermo during the period June–July, while two were of bovine origin, one from Palermo (23625) and one from Foggia (23078).

Resistance to antibacterial agents and heavy metals

Bacterial susceptibility to a number of antimicrobial agents and heavy metals was ascertained as previously described (Bauer *et al.* 1966; Ericsson & Sherris, 1971; Anderson & Threlfall, 1974; Nakahara *et al.* 1977). The following antibiotics were tested: ampicillin (Ap), 10 μg; carbenicillin (Cb), 100 μg; cephalothin (Cf), 30 μg; chloramphenicol (Cm), 30 μg; colimycin (Cl), 10 μg; fosfomycin (Ff), 50 μg; gentamicin (Gm), 10 μg; kanamycin (K), 30 μg; nalidixic acid (Na), 30 μg; rifampicin (Ra), 30 μg; streptomycin (Sm), 10 μg; tetracycline (Tc), 30 μg; trimethoprim-sulfamethoxazole (Sxt), 25 μg. The following salts of heavy metals were tested by the agar dilution method (Nakahara *et al.* 1977): cadmium chloride, cobalt chloride, lead nitrate, mercury chloride, nickel nitrate, sodium arsenate and zinc acetate.

Colicinogeny

Colicin production was detected by the soft-overlay method using *Escherichia coli* strain K12 Row as an indicator organism (Ozeki, Stocker & Smith, 1962).

Plasmid transfer

Exponential phase cultures of donor and recipient (1:1) were mixed. The recipient was the rifampicin resistant *E. coli* strain BM13 J53.

After 3, 6, 12 and 24 h of incubation at 37 °C, samples were spread on MacConkey

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agar plates containing rifampicin (250 μg/ml) and either chloramphenicol or streptomycin (50 μg/ml).

Curing of plasmids

Treatment of cultures with ethidium bromide (Bouanchaud, Scavizzi & Chabbert, 1969), acridine dyes (Watanabe & Fukasawa, 1961) or SDS/high temperature (Hill & Carlisle, 1981) was used to obtain plasmid curing.

After 18 h of incubation, cells were spread on MacConkey agar plates and, after further overnight incubation at 37 °C, the colonies were randomly picked and examined for plasmid profile, or screened for the loss of antibiotic resistance by replica-plating on MacConkey agar containing chloramphenicol (30 μg/ml) or streptomycin (30 μg/ml). Simultaneously cultures grown without any curing agent were replicated to test for spontaneous loss of the resistance determinants.

Plasmid DNA analysis

The technique of Birnboim & Doly (1979) was used. The resulting DNA solution was submitted to electrophoresis (5 V/cm) on horizontal slab gels (14 × 11 × 0·7 cm) containing 0·7 % agarose type II (Sigma). An estimation of the molecular weight (MW) of the plasmids was obtained by comparing their electrophoretic mobilities with that of plasmids of known molecular weight. These included: RSF 1010 (5·5 MDa), RSF 2124 (7·4 MDa), Sa (25 MDa), RP4 (34 MDa), R1 (60 MDa), R62 (80 MDa), R40a (96 MDa) (Southern, 1979). Strains carrying plasmids for use as molecular weight standards were provided by E. M. Lederberg, Plasmid Reference Center, Stanford University. Plasmid DNA was recovered from low-melting-temperature agarose type VII (Sigma) as previously described (Maniatis, Fritsch & Sambrook, 1982).

Restriction enzyme cleavage analysis was carried out by Eco RI (Miles) under conditions described by the manufacturer. The final product of digestion was subjected to horizontal gel electrophoresis on 1·0 % agarose type II.

Mouse infection

S. dublin 23481, 23481p$^-$ (cured of its large plasmid), and 23625 strains were grown overnight in trypticase soy broth at 37 °C. The broths were centrifuged and the cells washed twice with sterile NaCl solution (0·9 g/100 ml) and re-suspended in 0·1 M-$NaHCO_3$ solution. A 0·1 ml portion of each bacterial suspension (4×10^4 c.f.u./ml of 23481 and 23625 strains, and 8×10^4 c.f.u./ml of 23481p$^-$) was injected by the intraperitoneal route into each of five female BALB/C mice, weighing about 20 g. The inoculum size was confirmed by doing serial dilutions and colony counts. Deaths were monitored up to 21 days after injection. Samples of livers and spleens were cultured on MacConkey's agar and in Selenite F broth (Terakado *et al.* 1983; Chikami, Fierer & Guiney, 1985).

RESULTS

The results of the study of the 60 *S. dublin* isolates are summarized in Table 1.

All isolates were susceptible to the heavy metals and none was colicinogenic.

The majority of the isolates made before 1984 were antibiotic susceptible, only five showing drug resistance: 10739 to tetracycline (MIC 31·2 μg/ml), 10868,

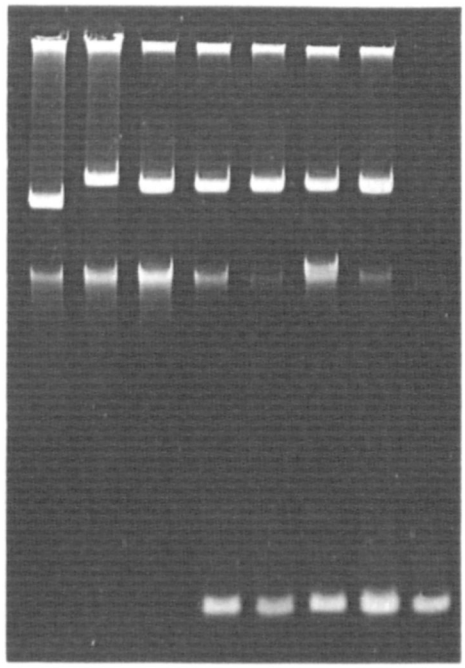

Fig. 1. Electrophoretic analysis of plasmid DNA of *Salmonella dublin* using 0·7% agarose. Lanes: 1 and 2, standard-molecular-weight plasmids (RP4 34 MDa and R1 60 MDa); 3, *S. dublin* 23078; 4, *S. dublin* 23625; 5, *S. dublin* 23490; 6, *S. dublin* 23501; 7, *S. dublin* 23481; 8, *S. dublin* 23481p (cured of its large plasmid).

11478, 13144 to chloramphenicol (MIC 62·5 μg/ml), and streptomycin (MIC 62·5 μg/ml) and 15007 to ampicillin (MIC 62·5 μg/ml), chloramphenicol (MIC 62·5 μg/ml), streptomycin (MIC 62·5 μg/ml) and tetracycline (MIC 31·2 μg/ml).

Twenty-four of the 25 *S. dublin* strains isolated during 1984 and the 3 made in 1985 were resistant to chloramphenicol (MIC 125 μg/ml) and streptomycin (MIC 62·5 μg/ml), while 1 isolate of bovine origin, 23078, was fully susceptible to antibiotics (Table 1).

Attempts to transfer the chloramphenicol and streptomycin resistance by conjugation to the recipient *E. coli* strain were unsuccessful after 3, 6, 12 and 24 h of incubation.

The *S. dublin* isolates during 1984 harboured one plasmid of approximately 50 MDa; all strains, except one, 23078, carried an additional plasmid of approximately 3 MDa (Fig. 1).

Furthermore, a spontaneous chloramphenicol susceptible derivative of 23481 strain was found to bear both plasmids.

The obscuring of plasmid bands by the chromosome DNA could be excluded by submitting to electrophoresis on 1·5% agarose the DNA from two strains, *S. dublin* 23481 and 23481 derivative, which was susceptible to chloramphenicol. In both cases the same plasmid pattern with no additional plasmid band was detected.

Restriction enzyme cleavage analysis of the 50 MDa and 3 MDa plasmids, after recovery from low-melting-temperature agarose, indicated high relatedness among the *S. dublin* isolates (Fig. 2).

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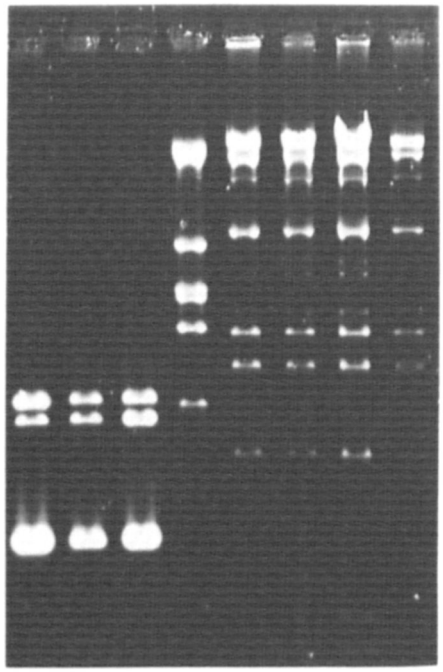

ig. 2. Restriction enzyme cleavage analysis of 50 MDa and 3 MDa plasmids from *Salmonella dublin* was carried out by ECO RI. Gel electrophoresis was conducted in 1% agarose at 20 V overnight. Lanes: 1, 2 and 3, small plasmid (*S. dublin* 23625, 23490, 23481); 4, lambda; 5, 6, 7, 8, large plasmid (*S. dublin* 23078, 23625, 23490, 23481).

To estimate genetic function of the 50 MDa plasmid, strain 23481 was cured of its plasmid by treatment with ethidium bromide. Biochemical reactions (API 20E), antigenic analysis and drug resistance pattern could not differentiate the 23481p$^-$ strain from the parental strain of *S. dublin*. 23481, 23481p$^-$ and 23625 strains were compared for virulence in mice infected by intraperitoneal injection. The *S. dublin* 23625 strain produced death in all of the mice within 1 week after injection; macroscopic necrotic lesions with abscesses were observed in their livers and spleens. All mice infected with *S. dublin* 23481 and 23481p$^-$ strains were killed 21 days after infection. Necrotic lesions were observed in the liver and spleen of the mice infected with *S. dublin* 23481, while no lesion was found in the mice infected with *S. dublin* 23481p$^-$, although *S. dublin* could be cultured from their spleens and livers after enrichment for 18 h in Selenite F broth. Several isolates of the 23481p$^-$ strain were re-examined for plasmid DNA content and they were shown to remain free of the large plasmid.

Attempts to cure *S. dublin* isolates of the small 3 MDa plasmid by ethidium bromide, acridine dyes and SDS/high temperature treatments were not successful, and no attempt was made to assign genetic functions to this plasmid.

Twenty-eight *S. dublin* isolates from 1971 to 1983 and the 3 made in 1985 showed a 50 MDa plasmid, and 7 of these possessed also a small plasmid identical in mobility to the plasmid of 3 MDa molecular weight of the 1984 isolates.

The Eco RI digestion pattern analysis revealed the high relatedness of these plasmids with those of the 1984 strain 23481.

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DISCUSSION

The majority of *S. dublin* isolates identified prior to 1984 were susceptible to antibiotics and carried a 50 MDa plasmid which encoded for virulence in mice. In contrast, all but 1 of the 28 isolates made from children and cattle during 1984 and 1985, together with a single isolate made in 1976, had similar characteristics. These included (*a*) chloramphenicol and streptomycin resistance, presumably chromosome encoded; (*b*) a 50 MDa plasmid associated with the virulence in mice; (*c*) an additional plasmid, of approximately 3 MDa, with no attributable function; (*d*) closely related restriction cleavage patterns of both 50 and 3 MDa plasmids.

The high similarity of antibiotic resistance pattern and plasmid profile of the isolates made during 1984 and 1985 from several geographically separated areas suggests possible dissemination of a single clone of *S. dublin*, although it was not possible to determine how this occurred from the available data.

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