EPIDEMIOLOGY OF INFECTION

Epidemiological analysis of strains of *Salmonella enterica* serotype Enteritidis from foodborne outbreaks occurring in Italy, 1980–1994

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Polymerase chain reaction (PCR-) ribotyping was performed on 243 strains of *Salmonella enterica* serotype Enteritidis isolated during the years 1980–1994 from 58 foodborne outbreaks occurring in different regions of Italy. The majority (37) of the outbreaks were attributed to phage type (PT) 4, followed by PT1 (seven outbreaks); the latter was identified in 1993 in Italy in epidemic strains of Enteritidis. In eight cases more than one phage type was recognised from a single event. Nine PCR-ribotypes (PCR-RTs) were detected, with a strong prevalence of PCR-RTs f7 and e5 (23 and 21 outbreaks, respectively). In two instances two distinct PCR-RTs were identified within strains from a single outbreak. All but one of the PT1 outbreaks were caused by PCR-RT f7, whereas PT4 outbreaks could be subdivided into six subsets. Clustering of isolates was consistent with data obtained from epidemiological investigations. PCR-ribotyping proved to be an effective and reliable tool for subtyping isolates of Enteritidis belonging to the most frequent phage types. Nevertheless, in terms of laboratory expertise and lack of inter-laboratory standardisation, this typing technique is best suited for reference laboratories.

Introduction

In the past 10 years the incidence of foodborne cases of infection caused by *Salmonella enterica* serotype Enteritidis has increased dramatically in many countries. Most cases of food-poisoning are related to the ingestion of raw or undercooked eggs or contaminated egg-containing foods [1, 2].

Phage typing has become the reference method for the primary subdivision of strains of this serotype for epidemiological investigations. In Europe, the predominant phage type (PT) is PT4, whereas in the USA and Canada the most frequently identified isolates are PT8 [3–5]. In Italy *c.* 80% of isolates belong to PT4 [6]. On this basis, phage typing alone appears to be inadequate for monitoring the spread of Enteritidis. Furthermore, changes in phage type occur by acquisition of a plasmid [7], loss of lipopolysaccharide [8] or phage conversion [9]. Likewise, interconversion of

PT4 and PT8 has been observed [9], even though epidemiological data confirm their stability under field conditions.

The application of polymerase chain reaction (PCR) ribotyping in molecular subtyping of epidemic and apparently sporadic strains of Enteritidis from Southern Italy has been reported previously [10]. This method uses PCR to amplify the 16S–23S intergenic spacer region of the bacterial rRNA operon. Fragment length polymorphisms identified by this technique have been used as an alternative genotyping approach to traditional ribotyping in the analysis of isolates of diverse bacterial species [11–13]. Within isolates of Enteritidis, the application of PCR ribotyping was able to identify multiple clones in the most frequent phage types [10].

In this study, 243 epidemic strains of Enteritidis isolated from 58 foodborne outbreaks occurring in Italy during 1980–1994 were submitted to phage typing and PCR ribotyping. The aim of the present work was to evaluate the effectiveness of this molecular typing method for strains isolated from food-poisoning events previously elucidated by epi-

demiological investigations carried out by Public Health Services.

Materials and methods

Bacterial strains

Two hundred and forty-three strains of Enteritidis from 58 food-poisoning outbreaks that occurred during the years 1980–1994 in Italy were examined. All available isolates from each outbreak were included in the study. In five and 24 cases, respectively, only isolates from food or from clinical cases were available for analysis. In 12 cases only one isolate was obtained.

Enteritidis strains from Southern Italy were referred to the Centre for Enterobacterial Pathogens of Southern Italy (CEPIM) by public health and hospital laboratories. Strains from Northern and Central Italy were provided by the Centre for Enterobacterial Pathogens of Northern Italy (CEPIS) and by the National Centre for Enterobacterial Pathogens (CNEP), Istituto Superiore di Sanità, Rome. Epidemic strains from Southern Italy had been analysed previously by phage typing and PCR ribotyping for comparison with a sample of non-outbreak strains isolated in the same period [10].

All isolates were identified and serotyped by standard techniques [14].

Phage typing

The strains were phage typed by the method of Ward *et al.* [15] without significant modifications. Isolates showing lysis patterns consistent with the recognised phage types were designated accordingly, whilst isolates with patterns not conforming to any of the currently identified types were classified as RDNC.

PCR ribotyping

Whole-cell DNA from each isolate was prepared by the technique described by Kostman *et al.* [12]. Briefly, pelleted bacterial cells from 1-ml overnight LB broth cultures were resuspended in 50 μ l of 10 mM Tris-HCl (pH 8.0)-1 mM EDTA and boiled for 5 min. Cells were then lysed with lysozyme 100 μ g/ml at 4°C for 15 min and proteinase K 100 μ g/ml at 55°C for 15 min. After heating at 95°C for 15 min, lysates were treated with RNAase 10 μ g/ml for 15 min at 37°C.

PCR genomic fingerprints were generated by the technique described by Kostman *et al.* [13] with minor modifications. Amplification reactions were accomplished in 25 μ l volumes containing 50 mM KCl, 10 mM Tris-HCl (pH 8.0), Triton X-100 0.1% v/v, 1.5 mM MgCl₂, 100 μ M each deoxynucleoside triphosphate, 25 pmol of each primer and 1.0 U of *Taq* polymerase (Promega, USA). Primers complementary to conserved regions of rRNA genes were 5'-

TTGTACACACCGCCC-GTCA-3' and 5'-GGTACCT-TAGATGTTTCAGTTC-3', respectively [13]. One μ l of each bacterial lysate was included as template. A negative control with 1 μ l of distilled water instead of the DNA sample was used in PCR amplification of each set of samples tested. The temperature cycles were as follows: 95°C for 5 min; 95°C for 1 min, 55°C for 1 min and 72°C for 1 min for 30 cycles; 72°C for 5 min [13].

The amplified DNA products were resolved by electrophoresis through acrylamide-bis-acrylamide (59:1) 10% w/v Tris-borate-EDTA gels containing glycerol 5% v/v and detected by silver staining [16]. The banding patterns on the gels were directly scanned to obtain migration distances and fragment sizes were calculated by the commercial software Gel Manager (BioSystematica, Tavistock, Devon). Fragments in the 615–1230-bp range were considered for the interpretation of PCR amplification patterns. Non-identical patterns were identified as unique PCR-ribotypes (PCR-RTs).

Reproducibility and stability of the patterns obtained had been previously assessed on representative isolates of Enteritidis by multiple PCR amplifications of the same lysate and multiple DNA preparations and PCR amplifications on repeated subcultures of the same isolate [10].

Results

The results obtained by phage typing and PCR ribotyping are summarised in Table 1. The characteristics of Enteritidis strains isolated from 36 of the 58 foodborne outbreaks studied are illustrated in Table 2. In 11 of the remaining 22 outbreaks, only one isolate was available from food or human cases and, in the remainder, two human isolates with identical phage types and PCR ribotypes were obtained.

Phage typing

Seven PTs were detected among the 243 isolates studied. Eight isolates were RDNC. Three food-poisoning outbreaks which occurred in the years 1980 and 1986 were associated with PT8, 37 which occurred since 1982 were caused by PT4, seven outbreaks which occurred in the years 1993 and 1994 were associated with PT1 and three with RDNC strains. In eight cases – outbreaks nos. 6, 8, 11, 13, 14, 15, 19 and 30 – different PTs were recognised within strains isolated from the same outbreak.

PCR ribotyping

Nine PCR-RTs were identified among the outbreak-related strains (Figs. 1 and 2). PCR-RTs a1 and d4 were identified in the strains isolated from three

Table 1. Results of phage typing and PCR ribotyping of 243 epidemic strains of *S. enterica* serotype Enteritidis isolated in Italy in the years 1980–1994

Year of isolation	Phage type	Number of isolates	PCR ribotype	Number of isolates
1980	8	4	a1	4
1982	4	6	e5	6
1986	4	5	i10	5
	8	1	d4	1
1989	4	17	f7	15
			e5	2
1990	4	4	f7	4
	1	1	f7	1
1991	4	33	f7	18
			e5	9
			b2	2
			d4	2
			h9	2
			f7	2
	RDNC	2	f7	1
	4/6/RDNC*	1	f7	1
	5a	1	f7	1
1992	4	31	e5	12
			b2	10
			f7	9
	1	5	f7	5
	RDNC	4	t19	2
			e5	1
			f7	1
	5a	2	e5	2
	7	1	e5	1
	30	1	f7	1
1993	4	31	e5	13
			b2	12
			u20	5
			h9	1
	1	18	f7	18
1994	4	56	e5	41
			f7	15
	1	17	f7	10
			e5	7
	RDNC	2	f7	2

*PTs 4, 6, RDNC or mixed lytic patterns were detected.

foodborne outbreaks that occurred in the years 1980 and 1986, and PCR-RTs i10, t19, u20 and h9 were isolated from four outbreaks occurring in 1986, 1992 and 1993, respectively. Five, 21 and 23 outbreaks were caused by strains of Enteritidis belonging to PCR-RTs b2, e5 and f7, respectively. In two cases (outbreaks 10 and 11) more than one PCR-RT was identified among isolates from a single epidemic event.

Correlation between PT and PCR-RT

The three outbreak-related groups of strains of PT8 could be subdivided into two PCR-RT a1 and one PCR-RT d4, respectively.

The RDNC strains showed PCR-RT f7 in two cases and PCR-RT t19 in one case.

Among seven sets of isolates belonging to PT1, six were attributed to PCR-RT f7 and one to PCR-RT e5. This observation is consistent with the findings of previous work on isolates of Enteritidis from Southern Italy that showed a close relatedness between epidemic PT1 strains and PCR-RT f7 and, at the same time,

nine of 18 sporadic PT1 strains were identified as PCR-RT f7; the remaining nine consisted of four distinct PCR profiles, b2, d4, e5 and m12 [10].

Of the 37 PT4 foodborne outbreaks, 36 were subdivided by PCR ribotyping into six subsets: 17 were attributed to PCR-RT e5, 11 to PCR-RT f7, five to PCR-RT b2, and one each to PCR-RT i10, PCR-RT u20 and PCR-RT h9. Data from epidemiological investigations were consistent in almost all cases with clustering of strains as shown by PCR-ribotyping.

In one outbreak (no. 10) two different PCR-RTs were recognised: e5 was detected in one isolate from chicken eggs and d4 in Enteritidis strains from ice cream and human stool. Interestingly, the egg isolate shared the same PCR-RT e5 as that from chicken eggs involved in outbreak no. 9 which occurred at the same time in Reggio Emilia, Northern Italy. Epidemiological investigation had demonstrated that eggs implicated in the two outbreaks had been acquired from the same laying flock.

Outbreak no. 11 involved elderly patients and staff members of a nursing home (Table 2, i), home-assisted patients (ii) and guests of a hotel (iii). Epidemiological investigation had indicated egg-containing dishes provided by a common catering service as the presumed vehicle of infection, but phage typing identified three different types, so failing to confirm the attribution of the three events to a single bacterial clone. PCR ribotyping also yielded three different profiles, but isolates from the nursing home and from home-assisted persons were all attributed to the same clone of PCR-RT f7, although one was identified as PCR-RT e5, whilst strains from hotel guests shared a quite distinct PCR-RT h9. All the strains possessed the 38-MDa serotype-specific plasmid alone, except for the PCR-RT e5 isolate, which exhibited an additional plasmid of 67.7 MDa (data not shown).

Discussion

Various techniques have been used to subdivide Enteritidis strains within the predominant phage types. These include plasmid profiling [17, 18], IS200 typing [19], pulsed-field gel electrophoresis [20, 21] and ribotyping [18, 20–22]. Nevertheless, application of these techniques to strains belonging to PT4 or PT8, which have caused the majority of the outbreaks reported in Europe and in the United States, respectively, exhibited insufficient discriminatory power in many instances and consequently often proved to be unsuitable as effective epidemiological tools. On the other hand, these observations have been interpreted as support for the hypothesis of the clonal dissemination of pathogenic strains of Enteritidis.

PCR ribotyping was used in previous work on

Table 2. Characteristics of strains of *S. enterica* serotype Enteritidis isolated from 36 foodborne outbreaks occurring in Italy in the years 1980–1994

Outbreak no.	Year of isolation	Geographic origin	Epidemiological context*	Number of typed strains	Phage type	PCR ribotype	Source
1	1982	Agrigento, Southern Italy	f	6	4	e5	Human stool
2	1986	Caserta, Southern Italy	f	5	4	i10	Human stool
3	1989	Verona, Northern Italy (i)†	r	2	4	f7	Human stool
		(ii)†		1	4	f7	Human stool
		(iii)†		3	4	f7	Egg-based unspecified food
				2	4	f7	Human stool
4	1989	Teramo, Central Italy	f	2	4	f7	Cream pie
				1	4	f7	Human stool
5	1989	Teramo, Central Italy	r	1	4	f7	Lasagna
				2	4	f7	Human stool
6	1990	Massa Carrara, Central Italy	f	1	4	f7	Unknown food
				3	4	f7	Human stool
				1	1	f7	Human stool
7	1991	Cremona, Northern Italy	f	1	4	f7	Home-made ice cream
				3	4	f7	Human stool
8	1991	Reggio Emilia, Northern Italy	r	1	5a	f7	Omelette
				2	4	f7	Human stool
9	1991	Reggio Emilia, Northern Italy	r	1	4	e5	Chicken egg
10	1991	Reggio Emilia, Northern Italy	r	1	4	e5	Chicken egg
				1	4	d4	Home-made ice cream
				1	4	d4	Human stool
11	1991	Fidenza, Northern Italy (i)‡	r	1	4/6/RDNC§	f7	Human stool
				1	RDNC	f7	Human stool
				8	4	f7	Human stool
		(ii)‡		1	RDNC	f7	Human stool
				1	4	e5	Human stool
		(iii)‡		2	4	h9	Human stool
12	1991	Palermo, Southern Italy	f	1	4	e5	Tiramisu
				3	4	e5	Human stool
13	1992	Aosta, Northern Italy	h	1	30	f7	Human stool
				5	1	f7	Human stool
14	1992	Frosinone, Central Italy	f	1	7	e5	Human stool
				1	4	e5	Egg-based unspecified food
				2	4	e5	Human stool
15	1992	Teramo, Central Italy	r	1	RDNC	e5	Artichoke
				1	4	e5	Lamb meat
16	1992	Teramo, Central Italy	f	1	4	e5	Tiramisu
				1	4	e5	Human stool
17	1992	Genoa, Northern Italy	f	4	4	b2	Egg-based unspecified food
				4	4	b2	Human stool
18	1992	Genoa, Northern Italy	f	1	RDNC	t19	Tiramisu
				1	RDNC	t19	Human stool
19	1992	Modena, Northern Italy	h	1	4	e5	Tiramisu
				2	5a	e5	Chicken egg
				2	4	e5	Human stool
20	1992	Modena, Northern Italy	r	4	4	f7	Tiramisu
21	1992	Rome, Central Italy	r	1	4	f7	Sandwiches
				3	4	f7	Human stool
22	1993	Bergamo, Northern Italy	r	5	1	f7	Human stool
23	1993	Verona, Northern Italy	f	1	1	f7	Home-made mayonnaise
				2	1	f7	Human stool
24	1993	Colorno, Northern Italy	f	5	4	u20	Human stool
25	1993	Palermo, Southern Italy	f	1	1	f7	Home-made mayonnaise
				1	1	f7	Human stool
26	1993	Cosenza, Southern Italy	f	1	4	b2	Egg-based unspecified food
				10	4	b2	Human stool
27	1993	Cosenza, Southern Italy	f	1	4	e5	Egg-based unspecified food
				11	4	e5	Human stool
28	1993	Messina, Southern Italy	r	3	1	f7	Tartines
				3	1	f7	Human stool
29	1994	Lentate sul Seveso, Northern Italy	r	5	4	e5	Vitello tonnato – home-made mayonnaise
				26	4	e5	Human stool
30	1994	Modena, Northern Italy	r	2	1	f7	Ice cream
				5	1	f7	Human stool
				2	4	f7	Human stool

Table 2. continued

Outbreak no.	Year of isolation	Geographic origin	Epidemiological context*	Number of typed strains	Phage type	PCR ribotype	Source
31	1994	Frosinone, Central Italy	r	7	1	e5	Human stool
32	1994	Livorno, Central Italy	r	1	1	f7	Pastries
				2	1	f7	Human stool
33	1994	Milan, Northern Italy	f	5	4	e5	Human stool
34	1994	Teramo, Central Italy	f	1	4	f7	Sheep milk
				2	4	f7	Human stool
35	1994	Milan, Northern Italy	f	1	4	e5	Vitello tonnato
				1	4	e5	Human stool
36	1994	Enna, Southern Italy	r	3	4	f7	Ice cream
				7	4	f7	Human stool

*h, hospital; f, family; r, food processing and catering establishment.

†Consecutive outbreaks in the same restaurant.

‡The three foodborne events were attributed to foodstuffs provided by a single catering kitchen.

§PTs 4, 6, RDNC or mixed lytic patterns were detected.

Fig. 1. Polymerase chain reaction ribotypes (PCR-RTs) identified among epidemic strains of *S. enterica* serotype Enteritidis isolated in the years 1980–1994. Lanes: 1 and 11, mol. wt marker 123-bp ladder (Gibco-BRL); 2, PCR-RT a1; 3, PCR-RT b2; 4, PCR-RT d4; 5, PCR-RT e5; 6, PCR-RT f7; 7, PCR-RT h9; 8, PCR-RT i10; 9, PCR-RT t19; 10, PCR-RT u20.

Enteritidis strains isolated in Southern Italy in the years 1980–1994 and was able to detect multiple clones within the most frequent phage types [10]. In the present study, analysis of PCR ribotypes of isolates from 58 foodborne outbreaks occurring in different regions of Italy in the same period further confirmed the usefulness of this molecular method to

monitor the epidemic spread of Enteritidis. In particular, identification of distinct patterns among PT4 strains appeared to be effective in grouping epidemiologically linked strains and in characterising otherwise undistinguishable outbreak isolates. Moreover, seven of eight outbreaks from which different phage types had been identified proved to be related

Fig. 2. PCR-amplification patterns of *S. enterica* serotype Enteritidis. Lanes 1–11, as for Fig. 1.

to a single bacterial strain. Phage type heterogeneity observed in such instances presumably depends on phage conversion phenomena [9].

In our experience PCR ribotyping is a simple and reliable technique that allows the rapid generation of molecular fingerprints and is also suitable for large-scale screening of bacterial isolates for epidemiological monitoring purposes. Nevertheless, the requirements in terms of availability of equipment and skill of personnel and lack of inter-laboratory standardisation suggest that PCR ribotyping should preferably be performed in specialised reference laboratories

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