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Molecular fingerprinting defines a strain of *Salmonella enterica* serotype Anatum responsible for an international outbreak associated with formula-dried milk

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SUMMARY

Molecular analyses based on plasmid profile typing and pulsed-field gel electrophoresis have defined a strain of *Salmonella enterica* serotype Anatum associated with the consumption of a particular brand of formula-dried milk responsible for an outbreak in late 1996/early 1997 involving 15 infants and 2 relatives in the UK, and 2 infants in France. The study has demonstrated the value of laboratory-based surveillance involving identification of the outbreak strain at the molecular level coupled with food microbiology and targeted epidemiological investigations, and has highlighted the importance of rapid communication and subsequent international collaboration through the European Union-funded Salm-Net salmonella surveillance network.

INTRODUCTION

In January 1997 the Laboratory of Enteric Pathogens (LEP) of the Public Health Laboratory Service (PHLS) of England and Wales recognized that since November 1996 there had been an increase in the number of isolates of *Salmonella enterica* serotype Anatum from infants aged 1–11 months in England and Wales, with 8 infections being identified in the period 5 November 1996–21 January 1997. The Scottish Centre for Infection and Environmental Health also reported four cases of *S.* Anatum in infants in the period October 1996–January 1997 [1]. The ages of these cases suggested the involvement of a baby food product and within 24 h of the increase being recognized a case-control study was initiated by the Communicable Disease Surveillance Centre (CDSC) of the PHLS. The results implicated a particular brand of formula-dried milk which had been consumed by 10 of the 12 infants in the study who had been infected with *S.* Anatum compared with 3 of 40 control infants [2]. By the end of January 1997, of 52 isolates of *S.* Anatum received in LEP in the 13-month period 1 January 1996–31 January 1997, 15 were from infants and 2 were from relatives of cases. Through the European Union (EU)-funded international salmonella surveillance network (Salm-Net) [3], an outbreak notification was sent electronically to collaborators in all participating countries. As a result of this notification 4 recent strains of *S.* Anatum from infants in France were referred to LEP for comparison with the putative UK epidemic strain [2]. On 20 February 1997 the Food Hygiene Laboratory (FHL) of the PHLS isolated a strain of salmonella, subsequently identified as *S.* Anatum by

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LEP, from a hitherto unopened packet of the product. Although this was 4 weeks after the identification of the outbreak, the product from which the strain of *S.* Anatum was isolated had been manufactured in October 1996.

For epidemiological investigations molecular fingerprinting of the putative outbreak strain was urgently required. We now describe the combined use of antibiogram analysis, plasmid profile typing and pulsed-field gel electrophoresis (PFGE) for defining the strain of *S.* Anatum responsible for the 1996/97 international outbreak in infants associated with the consumption of formula-dried milk.

MATERIALS AND METHODS

Bacterial strains

Seventy strains of *S.* Anatum isolated in the UK, 67 from humans, 2 from raw milk and 1 from the product, and 4 strains isolated in France were studied. Their sources and year of isolation are summarized in Table 1. All strains from humans were received in the period 1 January 1994–31 January 1997. The strains from raw milk were received in 1996 and the isolate from formula-dried milk product implicated by the case-control study was made by FHL on 20 February 1997. The strains of *S.* Anatum from France were isolated in late 1996 and were received by LEP in February 1997.

Serotyping, antibiogram and plasmid transfer

Strains were identified as *S.* Anatum by standard methods [4] and screened for resistance to ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulphonamides, tetracyclines, trimethoprim, furazolidone, nalidixic and ciproflaxin using an agar dilution method in Isosensitest agar [5]. For strains with demonstrable resistance to antibiotics, the transfer of resistance to a plasmid-free, nalidixic acid-resistant (*nal*^{r}) recipient strains of *Escherichia coli* K12 (= 14R525) was attempted by standard methods [6].

Plasmid profile and pulsed-field gel electrophoresis (PFGE)

Plasmids were extracted by the method of Kado and Liu [7] and sized in kilobases (kb) in relation to reference plasmids carried in a standard strain of *E. coli* K12, 39R861 [8]. Plasmid profile types (PPTs) were designated as PPT 0 (plasmid-free) and for plasmid-carrying strains, from PPT A through to PPT

Table 1. Salmonella enterica *serotype Anatum: antibiogram, plasmid profile types and pulsed-field profiles*

Country	Source	Year	Number studied	Antibiogram*		Plasmid profile types (PPTs)				Pulsed-field profiles (PFPs)		
				R-type	Number	E	Ei	Eii	Others	Number studied	PFP X5	Others
UK	Human	1994	18	$	18	0	0	0	18 (7 PPTs)	5	0	5 (5 PFPs)
		1995	6	Su	1	0	0	0	1 (1 PPT)	0	—	—
				$	5	0	0	0	5 (1 PPT)	2	0	2 (2 PFPs)
		1996	28	Fu	1	0	0	0	1 (1 PPT)	0	—	—
				$	27	5	1	0	21 (12 PPTs)	14	6†	8 (6 FPFs)
		1997 (to January 31)	15	$	15	11	0	0	4 (2 PPTs)	14	11	3 (2 PFPs)
	Raw milk	1996	2	$	2	0	0	0	2 (1 PPT)	2	0	2 (1 PFP)
	Dried milk‡	1997	1	$	1	1	0	0		1	1	0
France	Human	1997	4	ASu	1	0	0	1	—	1	1†	0
				$	3	1	0	0	2 (1 PPT)	3	1	2 (2 PFPs)

* Drug resistance symbols: A, ampicillin; Su, sulphonamides; Fu, furazolidone.
† Additional plasmid-derived fragments in isolates with additional plasmids – PPTs Ei and Eii (see text).
‡ Formula-dried milk (see text).

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N and PPTs P and Q. Four subtypes were also designated – PPTs Ai and Aii, and Ei and Eii. For the isolate for which the transfer of resistance was demonstrated (see below), the plasmid profile of the resistance plasmid-carrying recipient strain was compared to that of the drug-resistant donor strain of *S*. Anatum.

For pulsed-field gel electrophoresis (PFGE) the method for the preparation of DNA was a modification of that described by Powell and colleagues [9]. Following digestion with *Xba*I linearized fragments were resolved using the CHEF DR II system (Biorad UK Ltd) at 5·4 v/cm for 40 h, with pulse times of 5–60 sec. Fragments were sized in relation to a lambda 48·5 kb ladder (Sigma), and as a method control 2 strains with known pulsed-field profiles (PFPs) were included – *S*. Agona PT 15, PFP type *Sag*X6 [10] and *S*. Urbana P165191, PFP type *Surb*XI (this study). The PFPs of *S*. Anatum were designated in order of strain isolation as *San*PFP X1 through to *San*PFP X8.

RESULTS

Antibiogram and transfer of drug resistance

Of the 70 UK strains, 1 was resistant to sulphonamides (Su) and 1 to furazolidone (Fu) (Table 1). Of the 4 French isolates, 1 was resistant to ampicillin and sulphonamides (ASu). The remaining 71 isolates were all drug-sensitive.

For the isolate from France resistant to ampicillin and sulphonamides, these determinants were transferable to 14R525 (*E. coli* K12 *nal*^{r}) as a single linkage group. In contrast, the transfer of resistance to neither sulphonamides nor furazolidone was detected in the 2 UK isolates resistant to these antimicrobials.

Plasmid profile

Forty-seven of the 74 strains of *S*. Anatum (64%) possessed plasmids and 17 PPTs were identified (Table 2). With 2 exceptions isolates from the putative outbreak and from the formula-dried milk product were all characterized by a single plasmid of 72 kb (= PPT E). The 2 exceptions were a drug-sensitive isolate made in December 1997 from an infant in the UK, which possessed 2 additional plasmids of 65 and 58 kb (= PPT Ei), and the ampicillin and sulphonamide-resistant isolate from an infant in France which possessed an additional plasmid of 101 kb (Asu) (= PPT Eii). Subsequent resistance transfer experiments followed by plasmid profile analysis demonstrated that the 101 kb plasmid in this strain conferred resistance to ampicillin and sulphonamides.

Table 2. Salmonella *Anatum: plasmid profile types*

Plasmids (kb)	PPT	Number
—	0	27
33, 5·1	A	7
5·1	Ai	2
5·1, 4·9	Aii	1
72, 33	B	1
5·8, 5·5, 3·8	C	1
10, 6·0, 1·4	D	1
72	E	18
72, 65, 58	Ei	1
101, 72	Eii	1
29, 5·8, 4·3	F	1
86, 52, 5·5	G	1
4·3	H	1
4·0	I	1
< 2·9	J	1
5·8	K	1
86	L	2
2·9	M	3
12, 5·8, 5·2	N	1
86, 5·8, 5·2	P	1
65	Q	1

PPT, plasmid profile type.

For strains isolated in the UK PPTs E and Ei were not identified until late 1997 and were only identified in isolates of *S*. Anatum from infants who had consumed the implicated product (15 isolates), or from a sibling (1 isolate), a parent (1 isolate), or from the product (1 isolate). For the 4 strains of *S*. Anatum from France 2 isolates were plasmid-free (PPT 0), 1 possessed only a 72 kb plasmid (= PPT E) and the remaining strain possessed the 72 kb plasmid and an additional Asu drug resistance plasmid of 101 kb (see above).

The distribution of the most common PPTs from 1994 to 1997 is summarized in Table 3. Plasmid-free strains (PPT 0) were common throughout the study period and in 1995 and 1996 PPT 0 comprized the predominant type. The plasmid profile type PPT M, characterized by a single plasmid of 2·9 kb was identified in 1996 in 2 strains of *S*. Anatum from raw milk and subsequently in a single isolate from a patient.

Pulsed-field gel electrophoresis

Eight *Xba*I-generated PFPs designated *San*PFP X1 through to PFP X8 were identified in 42 isolates of *S*. Anatum studied by PFGE. Examples of *San*PFPs X1,

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Table 3. Salmonella *Anatum: distribution of predominant plasmid profile types, 1994–7*

Country	Source	Year	Number studied	PPT	Number	Per cent
UK	Human	1994	18	0	5	28
				A	7	39
				Ai	2	11
				Aii	1	6
				Others	3 (3 PPTs)	17
		1995	6	0	6	100
		1996	28	0	11	39
				E	5	18
				Ei	1	3
				Others	11 (11 PPTs)	39
		1997 (to January 31)	15	0	3	20
				E	11	73
				M	1	7
	Raw milk	1996	2	M	2	100
	Dried milk*	1997	1	E	1	100
France	Human	1997	4	0	2	50
				E	1	25
				Eii	1	25

* Formula-dried milk (see text).

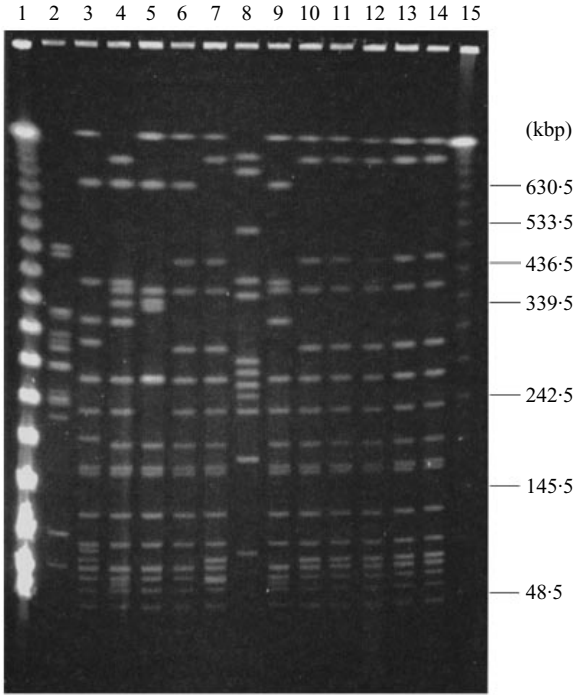

Fig. 1. PFGE profiles of *Xba*I-digested genomic DNA from isolates of *Salmonella* Anatum from infections associated with formula-dried milk.
Legend: Tracks 1–15 contained: 1 and 15, lambda 48·5 kb ladder (Sigma); 2, *Sag* PFP X6 (control strain, [10]); 3, *San* PFP X1; 4, *San* PFP X2; 5, *San* PFP X3; 6, *San* PFP X4; 7, *San* PFP X5 + additional plasmid-related fragment of approximately 80 kb [see text]; 8, *Surb* X1 (control strain); 9, *San* PFP X6; 10–14, *San* PFP X5. PFGE conditions: 180 V (5·4 V/cm); 40 h; Ramp, 5–60 s.

X2, X3, X4, X5 and X6 are shown in Figure 1. Eighteen isolates from the putative outbreak, including isolates from infants in the UK and France and from the product had a distinctive PFP which has been designated *San*PFP X5. The 2 outbreak-associated isolates with additional plasmids (PPTs Ei and Eii) possessed additional fragments of approximately 80 kb (PPT Ei) and 110 kb (Eii). However, in all other respects their PFPs were identical to *San*X5 and it is presumed that the additional fragments in these two isolates were plasmid-related.

DISCUSSION

The outbreak of *S.* Anatum infection described above was recognized by LEP in late January 1997 and the results of a case-control study implicated a particular brand of formula-dried milk. A precise definition of the putative outbreak strain was urgently required to support the on-going epidemiological investigations and the method of choice was plasmid profile typing combined with pulsed-field gel electrophoresis (PFGE). The value of plasmid profile typing for subdivision within serotype is well established [11] and since 1995 PFGE has proved useful for subdivision both within serotype and phage type in several outbreaks [10, 12–13]. In this outbreak molecular analyses based on plasmid-profile typing and PFGE demonstrated that the putative outbreak strain was

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defined by carriage of a plasmid of 72 kb (= PPT E) and by a particular pulsed-field profile (PFP 5). This plasmid and pulsed field profile combination was found only in isolates of *S.* Anatum associated with the outbreak, or in the strain from the product. In contrast over 50 strains which were not associated with the putative outbreak neither carried the 72 kb plasmid nor belonged to PFP type *San*X5. Additional plasmids were identified in 2 outbreak-related strains, 1 from an infant infected in the UK and 1 from an infant infected in France and additional fragments which were assumed to be plasmid-related were identified in the respective pulsed-field profiles. *In vivo* acquisition of plasmids from intestinal coliforms by drug-sensitive strains of salmonella has been demonstrated previously [14, 15], and it is possible that the presence of additional plasmids in two of the outbreak-associated isolates was the result of such acquisition.

As a result of the microbiological and epidemiological results the formula-dried milk product was withdrawn from the UK market on 24 January 1997 and from the French market on 8 February 1997. This study demonstrates the value of laboratory-based surveillance involving characterization of the putative outbreak strain by phenotypic and DNA-based methods, coupled with food microbiology and targeted epidemiological investigations. The study also provides an example of the value of the EU-funded Salm-Net salmonella surveillance network in the rapid notification of an outbreak to participating laboratories and subsequent collaboration at an international level.

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