The use of biochemical fingerprinting, phage typing and antimicrobial-susceptibility testing in the detection of epidemic strains of *Salmonella* of serotype Typhimurium in Iran

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Summary. A collection of 86 strains of *Salmonella* of serotype Typhimurium isolated from children with gastroenteritis in Tehran, Iran was examined for biochemical phenotype, phage type and antibiotic-resistance pattern. Twenty-seven biochemical phenotypes (BPTs), 14 discrete phage types (PTs) and 18 resistotypes (RTs) were identified. Fifty-three strains (62 %) belonged to two major and probably related BPTs, whereas the other 33 isolates belonged to less common BPTs. The two predominant BPTs contained 26 strains of the same PT and 23 strains of the same RT. Different PTs and RTs of strains with similar BPT were sometimes observed, possibly reflecting antibiotic pressures in Iran. These results suggest that two major “clones” of Typhimurium strains are particularly common in Iran and, although each method alone adequately detected these and other less common “clones”, biochemical fingerprinting provided additional information about relationships among strains.

Introduction

Strains of *Salmonella* of serotype Typhimurium are increasingly associated with outbreaks of food-borne disease worldwide.^{1–3} In Iran, Typhimurium was the major salmonella serotype isolated in a 3-year period.⁴ When typing antisera are available, identification of strains as Typhimurium rarely causes problems in routine clinical laboratories. However, in order to obtain a better understanding of its epidemiology, additional typing of Typhimurium strains will usually be required. The success of this goal depends on methods that discriminate and distinguish strains from different sources. Various techniques reported for typing Typhimurium strains include biotyping,⁵ phage typing,^{6,7} antimicrobial-sensitivity testing,⁷ plasmid analysis,^{9,10} multilocus-enzyme electrophoresis¹¹ and restriction-endonuclease analysis of chromosomal DNA.^{12,13} Because genetic variation in the typing characters of epidemic strains in the course of their epidemic spread may be expected, multiple typing is thought to provide a better picture of the “clonal” relationship among isolates.¹⁴

An automated typing method, based on biochemical fingerprinting (the PhP-system) reported elsewhere,^{15,16} gives a quantitative value of the metabolism of various substrates by measuring the speed and

intensity of each reaction. The “biochemical fingerprint” of each isolate is used to calculate with a personal computer similarities among strains tested with results shown in the form of a dendrogram. In another study,¹⁷ with a set of substrates specially chosen to subdivide Typhimurium strains, the system was shown to be highly discriminatory. In this study the PhP-system was used along with phage typing and antimicrobial testing to investigate relationships among epidemic strains of Typhimurium isolated from children with gastroenteritis in Iran.

Materials and methods

Bacterial strains

The 86 Typhimurium strains studied had been isolated from children < 5 years old with severe diarrhoea who attended children’s hospitals in Tehran, Iran, between Feb. 1985 and March 1986. Identification of strains and determination of antibiotic-resistance patterns have been described before.⁴ Strains were kept on nutrient-agar slopes at 4°C until tested for lysogenic pattern and biochemical phenotype.

Antibiotic resistance

Strains were tested against the antibiotics most commonly used in Iran, including chloramphenicol

Table I. Phage types, biochemical phenotypes and resistotypes of 86 Typhimurium strains isolated in Iran

Phage type	Number of strains	Biochemical phenotype	Number of strains	Resistotype	Number of strains
2NST	1	A1	3	388	1
3NST	1	A2	2	387	4
4	4	A3	2	386	1
5NST	7	A4	1	385	33
7NSTa	1	A5	2	380	15
7NSTb	3	A6	1	375	2
8	1	B1	43	373	1
8NSTa	1	B2	1	368	1
8NSTb	2	B3	1	365	2
11NSTa	8	B4	10	330	3
11NSTb	2	B5	1	285	9
18	1	B6	1	280	1
21	31	B7	2	275	1
22	9	B8	1	185	3
NT	14	B9	2	158	1
		B10	1	135	2
		B11	1	115	1
		B12	1	0	5
		C1	1		
		C2	1		
		C3	2		
		C4	1		
		C5	1		
		D1	1		
		E1	1		
		F1	1		
		G1	1		

(C), cotrimoxazole (SxT), ampicillin (A), tetracycline (T), kanamycin (K), nalidixic acid (Nx), streptomycin (S), cephalothin (Cf), and furaxone (F). Resistance to antibiotics was coded as follows: C = 200, T = 100, A = 50, SxT = 20, K = 10, S = 5, Cf = 2 and Nx = 1 with sensitivity to each antibiotic coded as 0. The sum of the values of the antibiotics to which strains were resistant was used to indicate the antibiotic resistotype (RT). Thus, an RT of 385 indicated a strain that was resistant to C, T, A, SxT, K, S whereas an RT of 0 indicated a strain that was sensitive to all antibiotics tested.

Phage typing

Strains were phage typed by the scheme of Lilleengen,¹⁸ exactly as described elsewhere¹⁷. As well as recognised phage-type patterns, some strains gave lytic patterns that were of non-specific types (NSTs) and other strains were not phage-typable (NT).

Biochemical fingerprinting

The principle of the PhP-system and the preparation of the plates have been described previously.^{15,16} Each isolate was subjected to 16 biochemical tests specially chosen to subdivide Typhimurium strains.¹⁷ The absorbance, A_{620} , of each reaction was measured after 4, 7, 24 and 48 h and the mean value of four readings was

calculated. Similarities among strains were calculated as correlation coefficients (r)¹⁵⁻¹⁷ and clustered according to the unweighted-pair group method with arithmetic averages (UPGMA)¹⁹ to yield a dendrogram. Strains with r values greater than the identity level (0.980) used in the PhP-system were assigned to the same biochemical phenotype (BPT). BPTs with > 1 isolate were called common BPTs and those with only one isolate were called single BPTs.

Results

The results obtained when isolates of Typhimurium were typed by each of the methods are summarised in table I. Eighty-one strains were resistant to between three and eight antibiotics and the most common RTs were 385 (33 isolates), 380 (15 isolates) and 285 (nine isolates); the latter RTs differed from RT 385 in sensitivity to S and T, respectively. Strains of these three RTs accounted for 66 % of the population tested. Only five isolates were fully sensitive to the antibiotics tested. In all, 18 different RTs were identified.

Seventy-two isolates were assigned to 14 discrete PTs and 14 were not typable (NT) (table I). The most common PTs were 21 (31 isolates), 22 (nine isolates), 11NSTa (eight isolates) and 5NST (seven isolates) which together accounted for c. 64 % of the strains.

Biochemical fingerprinting distinguished 27 BPTs with nine common and 18 single phenotypes. At the similarity level of 0.80, isolates grouped into seven

Figure. Grouping ("clones") of 86 Typhimurium strains by biochemical phenotype (BPT), phage type (PT) and antimicrobial-susceptibility pattern (RT).

clusters (figure). Strains in cluster A were characterised by their inability to ferment galactonic-lactone (galn-). Cluster B, which consisted of galn+ strains, accounted for 76 % of the isolates, with B1 (43 isolates) and B4 (10 isolates) dominating this cluster (figure); strains of B1 and B4 differed in their rate of trehalose fermentation. Cluster C, like cluster B, contained galn+ strains which, however, fermented tagatose slowly. Strains of BPTs D, E and F did not ferment inositol (ino-) or rhamnose (rha-) and strains of BPT F also failed to ferment galactonic-lactone. BPT G differed from the other strains in many biochemical reactions, including inositol and rhamnose (figure).

Biochemical fingerprinting together with phage typ-

ing yielded 41 "clonal" groupings (having the same BPT and PT) consisting of 14 common and 28 single phenotypes (table II). The dominant phenotype was B1/21 (23 strains); the closely related BPT B4 contained three strains of PT 21. A similar pattern was obtained when biochemical fingerprinting and resistotyping were used together (table III), with 46 BPT/RT groups (having the same BPT and RT) consisting of seven common and 39 single phenotype identified. The predominant BPT/RTs were B1/385 (19 strains), B1/380 (10 strains) and B1/285 (seven strains), strains of the latter differing from those of B1/385 only in resistance to S and T, respectively. Four strains of phenotype B4 also belonged to RT 385 (table III). The use of the three typing methods

Table II. Grouping of 86 Typhimurium strains isolated in Iran by biochemical phenotype and phage type

Common types		Number of isolates	Single types	
Biochemical phenotype	Phage type		Biochemical phenotype	Phage type
A1	NT	3	A2	NT
A3	NT	2		18
A5	11NSTa	2	A4	NT
B1	21	23	A6	NT
	22	5		
	11NSTa	3		
	8NSTb	2		
	7NSTb	2		
	5NST	5		
			B1	3NST
				2NST
				NT
			B2	22
			B3	21
B4	21	3		
	11NSTa	2		
	5NST	2	B4	7NSTb
				22
				11NSTb
			B5	21
			B6	11NSTb
B7	4	2	B8	21
			B9	21
			B9	22
			B10	11NSTa
			B11	7NSTa
			B12	21
			C1	22
			C2	NT
C3	NT	2	C4	4
			C5	8
			D1	NT
			E1	8NSTa
			F1	4
			G1	NT

together (figure) revealed eight common and 52 single "clonal" groupings with the BPT/PT/RT B1/21/385 being the most common (10 strains).

Discussion

These results suggest that there were two major, and probably related, "clones" of serotype Typhimurium causing gastroenteritis in Iran. Although each method alone adequately identified the presence of these and less common "clones", biochemical fingerprinting provided additional information about relationships among strains.

Resistotyping has been considered to be a relatively poor method for strain discrimination, mainly because antibiotic resistance in gram-negative bacteria is generally plasmid-encoded and, hence, strongly influenced by antibiotic pressure in the organisms' environment.

Table III. Grouping of 86 Typhimurium strains isolated in Iran by biochemical phenotype and resistotype

Common types		Number of strains	Single types	
Biochemical phenotype	Resisto-type		Biochemical phenotype	Resisto-type
			A1	0
				388
				368
A2	385	2		
A3	365	2	A4	385
			A5	380
				330
			A6	185
B1	385	19		
	380	10		
	285	7		
			B1	280
				386
				115
				275
				375
				389
				135
			B2	385
			B3	380
B4	385	4		
	380	3		
			B4	135
				388
				285
			B5	385
			B6	385
			B7	330
				185
			B8	388
			B9	385
				330
			B10	0
			B11	375
			B12	285
			C1	385
			C2	385
			C3	373
				385
			C4	388
			C5	0
			D1	158
			E1	0
			F1	185
			G1	0

Thus, acquisition of resistance genes by transposition from other plasmids carried by other bacteria in the host is very common.^{13,20} We have shown previously that the major RTs found among Typhimurium strains are also common among strains of other *Salmonella* serotypes isolated during a 3-year period in Iran.⁴ In contrast, phage typing has been considered as the method of choice for differentiating Typhimurium strains.^{13,21} Again, although the method is essential for primary subdivision of this serotype, it has proved insufficiently discriminatory in areas where a limited number of phage types predominate.²² Furthermore, it has been shown that carriage of R- and other factors by Typhimurium strains may restrict multiplication of infecting phages, reduce sensitivity, and result in interconversion of phage types of certain strains.^{23,24}

We have shown already that the frequency of resistance factors for the antibiotics used in this study varied between 20 and 83% among Typhimurium strains from Iran.⁴ Based on these observation, we postulated that variations observed in PTs or RTs of strains of the same BPT might reflect antibiotic pressure in Iran.^{4,25}

Barker *et al.*,²⁶ testing 2010 Typhimurium strains isolated from different sources over a 3-year period in Scotland, found that four major phage-type/biotype groups (epidemic strains) accounted for > 50% of their cultures. Our findings, based on combinations of BPT, PT and RT, also demonstrate that most of the Typhimurium strains isolated during 1 year in Iran belonged to a few major BPT/PT/RTs and support the claim of Anderson²⁷ that, in any country where conditions are favourable for the establishment of salmonellae in man and animals, relatively few clones are responsible for the majority of infections.

The main difference found in the biochemical reactions of the dominant BPTs B1 and B4 among our isolates was the rate of trehalose fermentation. Duguid *et al.*⁵ showed that Typhimurium strains isolated from different sources during the same outbreak had a stable biotype under natural conditions. However, they found that, after several years of storage, a few strains showed different patterns of trehalose fermentation and postulated that such differences were probably due to the selection of mutant strains during subculture. The strains tested in our study had also been stored for a long period before testing biochemical phenotypes. We previously evaluated, with the PhP-system, the stability of the biochemical phenotypes of enteropathogenic strains of *Escherichia coli* upon subculture and storage and observed that certain biochemical reactions of the bacteria changed slightly after subculture, storage or both.²⁸ Elsewhere,¹⁷ we

have reported similar changes in some biochemical reactions (including trehalose) of Typhimurium strains after subculture.

Biotyping of Typhimurium strains has delineated a special group of non-fimbriated (fim-), inositol and rhamnose non-fermenting strains termed "FIRN".²⁹ In an investigation of 2092 Typhimurium strains, Anderson *et al.*³⁰ found that 13% of strains belonged to the FIRN group, all of which were also D-xylose-negative. Based on genetical evidence,^{31,33} it has been suggested that FIRN strains have descended from a single clone (ino-, rha-, fim+) which has undergone successive mutations to fimbriation-negative and rhamnose fermentation-negative states. We did not investigate the presence of fimbriae among our ino-, rha- strains, but they fermented xylose slowly and belonged to phenotypes quite unlike others found in this study. Again, they differed from each other mainly in their galactonic-lactone reactions, suggesting that the ability to ferment galactonic-lactone (at least within the population tested in our study) may be an important biochemical character in tracing the origins of Typhimurium strains.

In conclusion, we found that strains of two major and probably related clones of Typhimurium were responsible for the majority of Typhimurium infections in Iran. Although each method detected these and other less common types in the population tested, biochemical fingerprinting by the PhP-system gave additional discrimination with regard to variety (number of phenotypes) and its ability to demonstrate possible clonal relationships among the strains.

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