MOLECULAR EPIDEMIOLOGY

An attempt to identify the evolutionary origin of a novel serotype of *Salmonella enterica* isolated from harbour porpoises

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The isolation since 1991 of a new serotype of *Salmonella enterica* (antigenic formula 4,12:a:-) from harbour porpoises (*Phocoena phocoena*) at post-mortem examination raised the question of its evolutionary origin. Representative strains of *S. enterica* serotype 4,12:a:- and strains of eight other serotypes of serogroup O4 with phase-1 flagellar antigen H 'a' were examined by *EcoRI* ribotyping, IS200 fingerprinting and PCR-based profiling. Statistical analysis of results of multiple typing showed that strains of *Salmonella* serotype 4,12:a:- were genetically distant from those of antigenically similar salmonella serotypes, none of which seemed likely to be the progenitor of the 'porpoise' serotype.

Introduction

Most serotypes of *Salmonella enterica* subsp. *enterica* (subsp. I) show diphasic variation of flagellar (H) antigens, the alternate expression of which is controlled by a complex switching mechanism [1]. However, certain salmonellae of clinical and epidemiological importance – such as serotypes Enteritidis (1,9,12:g,m:-) and Typhi (9,12,[Vi]:d:-) – express only one type of flagellar antigen, i.e., they are monophasic. These monophasic serotypes may represent ancestral types which never acquired a second flagellar gene, or the necessary switching mechanism, or they might have originated as mutant strains of diphasic serotypes that had lost either the switching mechanism or the ability to express a second flagellar antigen (usually, but not always, phase 2) [2]. The phylogenetic origin of some monophasic serotypes of *Salmonella* has been revealed by molecular typing methods [2]. For example, the monophasic *S. enterica* serotype 9,12:l,v:- of serogroup D1 that emerged in several countries was shown to have arisen from an ancestral strain of *Salmonella* of the diphasic serotype Goettingen [2].

Since 1991, isolates of a new monophasic serotype (antigenic formula 4,12:a:-) of *S. enterica* have been obtained at post-mortem examination from harbour

porpoises (*Phocoena phocoena*) caught accidentally in fishing nets or found stranded around the Scottish coastline [3]. This novel 'porpoise' serotype – which should be, but has not yet been, named – comprises at least two clonal groups [4], but information about its mode of transmission and evolutionary origin is lacking. In this study, strains of *S. enterica* of serotype 4,12:a:- and eight antigenically similar serotypes were examined by ribotyping, insertion sequence (IS200) fingerprinting and PCR-based profiling in an attempt to reveal the possible origin of the 'porpoise' serotype.

Materials and methods

Bacteria

The 10 serogroup O4 strains of *S. enterica* of subsp. I examined (Table 1) included: S932228 and S932732, representative strains of the two clonal lines of the 'porpoise' serotype 4,12:a:- [4]; and eight strains of other rare serotypes with phase-1 flagellar antigen H 'a' – Arechavaleta, Bispebjerg, Fulica, Hessarek, Huettwillen, Kisangani, Nakuru and Tinda [5]. All strains, including strain NCTC 7375 originally from the National Collection of Type Cultures (Central Public Health Laboratory, Public Health Laboratory Service, 61 Colindale Avenue, London), were from the collection of the Scottish Salmonella Reference Laboratory, Stobhill Hospital, Glasgow. Strains were maintained on Dorset's egg slopes at room temperature (18–20°C) until they were cultured on blood agar (Oxoid) incubated overnight in air at 37°C.

Table 1. Strains of *Salmonella* examined

Serotype	Strain no.	Antigenic formula*		
		O antigens†	H antigens	
			phase 1	phase 2
—‡	S932228	4,12	a	—
—	S932732	4,12	a	—
Arechavaleta	S/871826	4,[5],12	a	1,7
Bispebjerg	S/790913	<u>1</u> ,4,[5],12	a	e,n,x
Fulica	539K	4,[5],12	a	1,5
Hessarek	S/923437	4,12, <u>27</u>	a	1,5
Huettwillen	7455/90	<u>1</u> ,4,12	a	1,w
Kisangani	NCTC 7375	4,[5],12	a	1,2
Nakuru	3565	<u>1</u> ,4,12, <u>27</u>	a	z ₆
Tinda	267K	<u>1</u> ,4,12, <u>27</u>	a	e,n,z ₁₅

*See reference [5].

†O antigens that may be absent are designated as, e.g., [5] and those determined by phage conversion are underlined, e.g., 1, 27.

‡For ease of reference this new serotype, not yet named, is designated in the text as the 'porpoise' serotype.

Ribotyping and IS200 fingerprinting

One colony from a blood agar culture was inoculated in 10 ml of L broth [6] in a disposable 20-ml screw-capped plastic tube and incubated with shaking for 18 h at 37°C. Bacteria were pelleted by centrifugation for 30 min at 3000 *g* and resuspended for 20 min at

room temperature in formalin 10% v/v in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to inactivate cellular nucleases. Cells were washed four times in TE buffer to remove formalin. DNA was extracted by a cetyltrimethylammonium bromide miniprep method [7] and digested to completion with restriction endonucleases *Eco*RI or *Pst*I (*Pst*I does not cut within the IS200 sequence). Strains were examined for their ribotypes and IS200 fingerprints after Southern hybridisation of digested DNA fragments with appropriate digoxigenin (DIG)-labelled probes. Electrophoresis, Southern transfer, DNA probes and hybridisation conditions were as described previously [8]. Line diagrams, representing ribotypes and IS200 fingerprints, were prepared directly from bands present on X-ray films with Microsoft Draw for Windows. Given the difficulty of comparing the sizes of high mol.-wt bands, only those bands less than *c.* 9–14 kb were considered when assessing strain similarity.

PCR-based typing techniques

PCR fingerprints for all 10 strains were generated from supernates of boiled suspensions prepared directly from bacterial colonies [9]. PCR spacer ribotypes were determined as described previously [10]. RAPD-PCR

Fig. 1. Line diagram representing *Eco*RI ribotypes (lanes 2–11) of serotypes of *Salmonella* serogroup O4 with phase-1 flagellar antigen H 'a'. Mol. wt markers (kb) are indicated in lane 1.

profiling was performed with the 10-mer primer 1254 (5'-CCGCAGCCAA-3') [11] and the amplification conditions described by Hilton *et al.* [12]. Amplification products (7- μ l amounts) were separated by electrophoresis at 7–10 V/cm through Hi-Pure Low EEO agarose (Biogene, Kimbolton, Cambridge) 2% w/v in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA) containing ethidium bromide 0.5 mg/L.

Genetic relatedness of serotypes

Data from all typing methods were used to assess genetic relatedness among strains. Dendrograms were generated with the unweighted pair group mathematical average (UPGMA) in the Multivariate Statistical Package for Windows, version 3.1 (Kovach Computing Services, Pentraeth, Anglesey).

Results

Ribotyping and IS200 fingerprinting

When *Eco*RI-digested DNA of the 10 strains of *Salmonella* was hybridised with the ribosomal RNA gene probe, 10 ribotypes (E1–E10) were observed (Fig. 1, lanes 2–11). Ribotypes E1–E8 (Fig. 1, lanes 2–9) were obtained for strains of the eight named serotypes. Ribotypes E9 and E10 (Fig. 1, lanes 10 and 11) were

those shown by 'porpoise' strains S932732 and S932228, respectively. Although distinct, ribotypes E9 and E10 were related, sharing nine bands of *c.* 1.4, 2.2, 2.3, 2.7, 3.1, 3.8, 5.6, 7.4 and 9.3 kb. Ribotypes E1–E8 shared six or less of these nine bands.

When *Pst*I-digested DNA was hybridised with the IS200 probe, 10 fingerprints were identified (Fig. 2), including that of serotype Nakuru which carried no copies of IS200 (Fig. 2, lane 7). Strains of serotypes Huettwillen and Tinda carried five copies of IS200 (Fig. 2, lanes 5 and 8, respectively). Both strains of the 'porpoise' serotype carried five copies of IS200 and they shared hybridised fragments of *c.* 2.9 and 3.7 kb (Fig. 2, lanes 9 and 10). Strains of the other five serotypes examined contained between one copy (serotype Hessarek) and 13 copies of IS200 (serotype Bispebjerg) (Fig. 2, lanes 4 and 2, respectively). Molecular sizes estimated from different gels indicated the presence of a 5.4-kb band in the profiles of serotype Huettwillen and the 'porpoise' strain S932228 (Fig. 2, lanes 5 and 10, respectively).

PCR profiles

When the strains were analysed by PCR spacer ribotyping, two distinct fingerprints were obtained (data not shown). One profile, shown by strains of serotypes

Fig. 2. Line diagram representing IS200 profiles (lanes 1–10) of serotypes of *Salmonella* serogroup O4 with phase-1 flagellar antigen H 'a'. Mol. wt markers (kb) are indicated in lane 11.

Arechavaleta, Bispebjerg, Fulica, Nakuru, Tinda and both strains of the 'porpoise' serotype, comprised two bands of *c.* 700 and 800 bp; the other profile, shown by strains of serotypes Hessarek, Huettwillen and Kisan-gani, contained an additional band of *c.* 750 bp.

RAPD-PCR generated 10 distinct fingerprints for the 10 strains examined (Fig. 3, lanes 1–12). (Each of the lanes 1 and 2, and 9 and 10 shows preparations of the same strain.) Fingerprints of the 'porpoise' strains S932732 and S932228 were distinguishable but showed many common bands (Fig. 3, lanes 11 and 12, respectively).

Genetic relatedness of strains of antigenically related serotypes

Individual dendrograms generated from ribotypes, IS200 profiles and RAPD-PCR fingerprints confirmed that strains of the 'porpoise' serotype were themselves highly related at 75%, 50% and 90%, respectively (Fig. 4a–c). The 'porpoise' serotype was shown to be poorly related to the other eight serotypes by ribotyping (*c.* 40–65%), IS200 fingerprinting (0–30%) and RAPD-PCR profiling (*c.* 65–86%), respectively (Fig. 4a–c). A dendrogram based on data from all typing methods

confirmed the close relationship of the two 'porpoise' strains (79%), but showed that they were distantly related (*c.* 54–64%) to strains of the other eight serotypes examined (Fig. 5).

Discussion

Molecular typing has enhanced understanding of the epidemiology of salmonella infection by increasing knowledge of the genetic relationships among strains. For example, ribotyping, by consideration of polymorphisms in the highly conserved ribosomal RNA genes, has helped to identify sources and modes of transmission. Ribotypes of strains belonging to the same serotype have common features but differences allow strains responsible for outbreaks and sporadic cases of illness to be distinguished [13–15].

If, as has been argued, ribotypes correlate with multilocus enzyme electrophoresis profiles and reflect genetic lineages rather than short-term genetic evolution [16], they should be useful for the study of bacterial population genetics. In this study, ribotyping confirmed our previous finding [4] that strains of the recently discovered *Salmonella* serotype 4,12:a:- form

Fig. 3. RAPD-PCR fingerprints (lanes 1–12) of serotypes of *Salmonella* serogroup O4 with phase-1 flagellar antigen H 'a'. Lanes 1 and 2 (serotype Arechavaleta), and 9 and 10 (serotype Tinda) show different DNA preparations of the same strain. Mol. wt markers (kb) are indicated in lane 13.

Fig. 4. Dendrograms based on similarity coefficients from: **a**, *EcoRI* ribotyping; **b**, *IS200* profiling; **c**, RAPD-PCR fingerprinting of serotypes of *Salmonella* serogroup O4 with phase-1 flagellar antigen H 'a'. Dendrograms were constructed by the unweighted pair-group average method in the Multivariate Statistical Package, version 3.1.

a single cluster. However, they are no more closely related to serogroup O4 strains of antigenically related serotypes (i.e., with phase-1 flagellar antigen H 'a') than they are to strains of serotypes with other phase-1 flagellar antigens (data not shown).

The 708-bp *IS200* element has been found in a wide range of salmonellae but it is absent from some serotypes, such as *Agona* [17]. In theory, *IS200* can transpose to other genomic sites but rarely does so *in vitro* [18]. For that reason, common bands in the *IS200* profiles of different strains may indicate a common

Fig. 5. Dendrogram based on similarity coefficients from combined *EcoRI* ribotyping, *IS200* profiling and RAPD-PCR fingerprinting of serotypes of *Salmonella* serogroup O4 with phase-1 flagellar antigen H 'a'. The dendrogram was constructed by the unweighted pair-group average method in Multivariate Statistical Package, version 3.1.

origin [18] and give insight into the evolution of new serotypes [2]. The *IS200* profiles of 41 isolates of *Salmonella* serotype 4,12:a:- examined previously confirmed the findings from ribotyping – i.e., they showed the existence of two clonal groups [4]. In this study, results from *IS200* analysis also supported the conclusions of ribotyping, namely, that the 'porpoise' strains were poorly related to strains of antigenically related serotypes. Indeed, among the strains examined, only the strain of serotype Huettwillen shared a common band with one of the 'porpoise' strains.

PCR spacer ribotyping was poorly discriminatory, but it distinguished strains of serotypes Hessarek, Huettwillen and Kisangani from the other serotypes examined. Ribotyping – a powerful tool for establishing strain relatedness [16] – had already eliminated serotypes Kisangani and Hessarek as likely progenitors of the 'porpoise' serotype. The reliability of RAPD-PCR profiling and, accordingly, its value for the detection of similarities and differences between bacterial strains has been questioned [19]. However, when amplification conditions are carefully controlled, as they were in this study, profiles are reproducible and provide clear strain discrimination. The 'porpoise' strains are closely related (90%) but, as already shown by ribotyping and *IS200* profiling, none of the antigenically related serotypes examined was a likely progenitor of the 'porpoise' serotype.

Popoff and Le Minor considered that 'serotypes Fulica and Hessarek, with the same global antigenic formula, should not be combined because their biochemical characters are very different' [5]. Results from this study confirmed not only that these two serotypes are genetically distant but also that neither is particularly related to the 'porpoise' serotype (see Figs. 4 and 5). Another *Salmonella* as rare as serotype Fulica, and originally believed to have the same antigenic formula as the 'porpoise' serotype, is serotype Makoma [20]. However, its antigenic formula was subsequently

redefined as 1,4,[5],12, 27:a:e,n,x. The serotype name Makoma has since been withdrawn from the current scheme of recognised salmonellae [5] because it is a member of subsp. II. Serotypes in subsp. II, regardless of their antigenic similarity to the porpoise serotype, were not examined in this study because their biochemical properties are so very different from those of strains in subsp. I [5].

The discovery of *Salmonella* serotype 4,12:a:- in harbour porpoises was reported only recently [3]. Although epidemiological and phylogenetic information is lacking, it is possible to speculate on the possible evolutionary origins and routes of transmission of this new serotype. In a previous study, *Brucella maris*, a new species, was reported in various stranded marine mammals including porpoises and dolphins [21, 22]. The natural hosts of brucellae include cattle, goats, sheep and pigs, in which animals infection of the reproductive tract can lead to abortion or to male sterility [23]. The possible occurrence of widespread brucellosis among marine mammals led to theories about the origins of *B. maris*, namely, that the bacterium had been carried by the ancestors of modern dolphins or porpoises millions of years ago or that it had emerged recently from a source such as agricultural run-off contaminated with faeces of livestock animals.

Salmonella O4 strains with phase-1 flagellar antigen H 'a' are rarely isolated in the UK [24]. High concentrations of bacteria and viruses at marine sites around sewage outfalls are likely to facilitate exchange of genetic material [25], giving rise to variants of recognised microbial species that are adapted to cause infection in sea mammals. Thus, evolutionary convergence of antigenic phenotype and horizontal transfer and recombination of antigen genes between lineages rather than minor mutation may have given rise to this new porpoise-associated serotype of *Salmonella*. Whilst this study has failed to identify any of the rare serotypes examined as the likely progenitor of serotype 4,12:a:-, the findings support the idea that the 'porpoise' serotype is truly new.

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