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Levels of virulence are not determined by genomic lineage of *Salmonella enterica* serotype Enteritidis strains

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SUMMARY

Mouse virulence and the ability to adhere to, and invade cultured MDCK cells were investigated in 38 phage type reference strains of *Salmonella enterica* serotype Enteritidis and correlated with genomic lineage. The genomic lineage of 11 of the strains was determined in the present study; one IS*200* and one ribotype pattern that had not been reported previously were observed. Log c.f.u. in the spleen 10 days post intraperitoneal (i.p.) infection with 3×10^3 bacteria (logVC10) varied between 2·9 and 8·7. The reference strains of PT7 and PT23 were found to be semi-rough and were of low virulence. All other strains possessed smooth LPS. Within each of the two major clonal lines, as well as among phage types outside these, both highly virulent and moderate to low virulent strains were present. While all strains of PT1, PT2 and PT8 were highly virulent, low virulent strains were detected in PT4 and PT13. The ability to adhere to, and invade MDCK cells varied between phage types (adherence between 13 and 61% of the inocula and invasion between 4 and 151% of the adherent cells). The results of the cell culture experiments did not correlate with the results of mouse virulence tests. No correlation between clonal lineage and virulence was found within *S.* Enteritidis. It seems most likely that some strains have lost some of the essential factors enabling this serotype to cause successful systemic infection.

INTRODUCTION

Salmonella enterica serotype Enteritidis is the most common cause of human salmonellosis in many countries [1], often associated with consumption of eggs and poultry products [2, 3]. In chickens, *S.* Enteritidis does not normally cause disease, although internal organs, such as liver, spleen, heart and gallbladder may be colonized at a low level for a short period. Of particular significance to the epidemiology of human infections is the fact that the intestine and cloaca of infected birds remains culture-positive for a prolonged period, and that oviduct tissues may be colonized [4], increasing the risk of crop contamination during slaughter and the production of shell-contaminated, as well as internally contaminated eggs.

Strains of *S.* Enteritidis may differ with respect to virulence [5–7]. Within phage type 4, which has been dominating in Europe [1], virulence variation has been linked to the possession of a virulence plasmid [8, 9], as well as to tolerance of a number of environmental conditions [10]. Recently, strains isolated from clinical cases were shown to have lower LD_{50} in chicken than strains isolated from the environment or from food products [11].

From a public health point of view, it is essential to be able to differentiate between virulent and avirulent

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Table 1. *Mouse virulence and LPS-profile of strains of* S. *Enteritidis in relation to clonal line*

Strain	Phage type	Clonal line*	LPS R/S†	Ribotype‡	LogVC10	x̄LogVC10
E2341	1	I	S	I	8·3 ± 1·3	7·5 ± 1·4**
P66040	3	I	S	III	8·3 ± 0·4	
E2187	4	I	S	II	8·3 ± 0·7	
P99764	4a	I	S	II	8·0 ± 1·3	
P70001	5	I	S	I	8·5 ± 0·5	
Ent5a	5a§	I	S	II	n.d.	
P99327	6	I	S	II	5·1 ± 0·9	
E2408	6a	I	S	I	5·7 ± 0·6	
E1492	7	I	R	II	4·6 ± 0·3	
E2402	9	I	S	II	8·7 ± 0·3	
Ent9a	9a§	I	S	IX	n.d.	
P95661	12	I	S	II	7·0 ± 2·1	
Ent11A	11a§	I	S	III	n.d.	
P95940	17	I	S	II	7·9 ± 0·8	
E1949	19	I	S	III	8·5 ± 0	
Ent20a	20a§	I	S	II	n.d.	
P72580	21	I	S	II	8·3 ± 1·3	
P100613	25	I	S	II	7·2 ± 2·0	
Ent31	31§	I	S	II	n.d.	
Ent32	32§	I	S	I	n.d.	
Ent33	34§	I	S	II	n.d.	
E2452	2	II	S	II	8·7 ± 0·3	7·0 ± 2·3**
E2468	8	II	S	II	8·5 ± 0	
E3945	10	II	S	III	4·0 ± 0·2	
E464	13	II	S	II	7·6 ± 2·1	
Ent13A	13a§	II	S	VIII	n.d.	
P84357	22	II	S	II	8·4 ± 0·2	
P88255	23	II	R	II	3·5 ± 1·4	
P99768	24	II	S	II	8·5 ± 0	
Ent28	28†	II	S	II	n.d.	
Ent9b	9b§	Other	S	II	n.d.	6·6 ± 2·8
E2109	11	Other	S	I	8·3 ± 0·8	
E2384	14	Other	S	IV	2·9 ± 1·2	
Ent14b	14b§	Other	S	II	n.d.	
E2400	15	Other	S	I	8·5 ± 0	
E866	16	Other	S	VII	3·0 ± 1·4	
P89448	18	Other	S	II	8·5 ± 0	
P68147	20	Other	S	I	8·5 ± 0	

* Clonality defined based on IS*200* typing according to Stanley and colleagues (1991) with grouping according to Olsen and colleagues (1994).

† S, smooth; R, rough LPS profile.

‡ *Sma*I ribotype with probe complementary to 16S and 23S rRNA.

§ IS*200* and ribotyping results from this study.

** x̄LogVC10 without the rough strains of PT7 and PT23 were 7·2 ± 1·2 for clonal line I and 7·6 ± 1·8 for clonal line II.

strains, preferably without the use of animal models. *S*. Enteritidis contains more than one clonal line as identified by multi locus enzyme electrophoresis (three lines) [12], the copy number and the location of insertion sequence IS*200* (at least 3 lines) [13, 14], and typing with a variety of DNA based typing methods (two major and several minor lines) [14]. In the present study, mouse virulence of strains of *S*. Enteritidis from different clonal lines was compared with adhesion and invasion in cell culture in order to evaluate whether levels of virulence could be predicted by the use of cell culture, and to determine whether virulence was influenced by genomic lineage of the *S*. Enteritidis strains.

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MATERIALS AND METHODS

Bacterial strains

The 38 phage type (PT) reference strains of *S.* Enteritidis used are listed in Table 1. They consisted of 27 strains selected based on reports on genomic typing results [14] and 11 page type reference strains which had not previously been allocated to a clonal line. The strains were kindly provided by Dr L. R. Ward, Central Public Health Laboratory (CPHL), London, UK. For PT1, PT2, PT4, PT8 and PT13, additional wild-type strains were included to test the generality of the results obtained by challenge of mice (Table 2). These strains were kindly provided by Dr E. J. Threlfall, CPHL, London, UK. *S.* Typhimurium 13/74 and its isogenic *invH*$^{-}$ mutant [15] were used as positive and negative controls in tissue culture experiments. Strains were cultured and maintained as previously reported [14].

Table 2. *Variation of virulence within selected phage types of* S. *Enteritidis*

Phage type	Strain	LogVC10	$\bar{x}$LogVC10
PT1	E2341	8·3 ± 1·3	8·8 ± 0·6
	P79582	9·4 ± 0·3	
	P82987	8·7 ± 0·5	
PT2	E2452	8·7 ± 0·3	8·6 ± 0·1
	P79578	8·5 ± 0	
	P88343	8·7 ± 0·3	
PT4	E2187	8·3 ± 0·4	7·3 ± 1·7
	P49120	5·7 ± 1·6	
	P132344	6·5 ± 0·6	
	P125592	8·5 ± 0	
PT8	E2468	8·5 ± 0	8·6 ± 0·1
	P87975	8·5 ± 0	
	P87888	8·7 ± 0·6	
	P127527	8·5 ± 0	
PT13	E464	7·6 ± 2·1	7·2 ± 2·1
	P106547	8·5 ± 0	
	P143029	8·5 ± 0	
	P141006	4·2 ± 0·9	

Allocation of strains into clonal lines based on genotype

Strains were allocated to a clonal line by the method of Stanley and colleagues [13] based on IS*200* profiles using the restriction enzymes *Pvu*II and *Pst*I (Boehringer–Mannheim), and further characterized by ribotyping using the enzyme *Sma*I. Isolation of DNA, restriction endonuclease digestions, agarose gel electrophoresis, preparation of probes, Southern analysis, detection of hybridization results and allocation to IS*200*-type and ribotype were carried out as previously reported [14].

Profiling of LPS

LPS molecules were selectively isolated by the proteinase K method, separated by SDS–Page and demonstrated by silver staining as previously reported [16].

Mouse virulence

Strains were tested for mouse virulence following intraperitoneal challenge of groups of five 21 g female Balb/C mice with suspensions made from overnight cultures and containing 3×10^3 c.f.u. in Brain Heart Infusion broth (BHI) (Oxoid) as previously reported [17]. Five mice, which received BHI only, served as controls. These were all negative for salmonella upon the termination of the testings. The $\log_1$ c.f.u. in spleens 10 days post infection (LogVC10) was used as a measure of virulence. For statistical purposes, mice that were killed in the terminal stages for animal welfare reasons were allotted a value of 8·5, provided the spleen contained a pure culture of salmonella. The detection level of the assay was 10^2 c.f.u. per spleen. The minimum logVC10 was therefore taken as 2·0.

In vitro cell assays

The ability of the bacteria to adhere to, and to invade cultured MDCK (Manine-Darby Canine Kidney) cells was determined essentially as reported by Fields and colleagues [18]. A detailed description of methods used has been given elsewhere [17].

Statistics

LogVC10 values and c.f.u. associated with cell monolayers were compared by Bonferroni's pairwise comparison of means using the computer software Statistix version 3.

RESULTS AND DISCUSSION

Allocation of strains into clonal line

Eleven of the phage type reference strains had not previously been allocated to a clonal line. These

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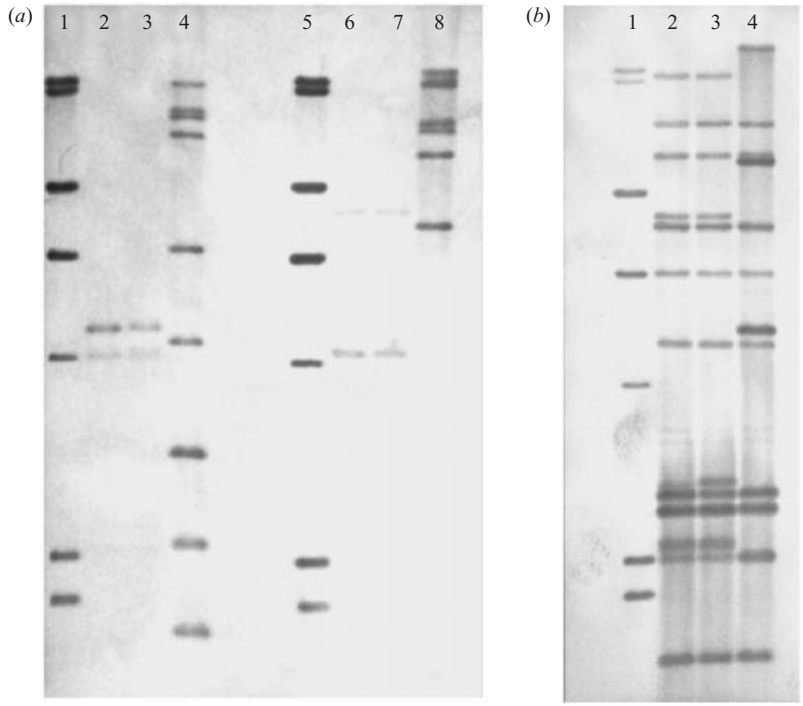

Fig. 1. IS*200* and ribotype patterns that had not previously been demonstrated among strains of *S*. Enteritidis. (a) IS*200* patterns. Lanes 1, 5: molecular size marker phage lambda *Hin*dIII; lanes 2–4 show *Pst*I-IS*200* patterns: 2, 3: the commonly observed pattern I (strains of PT1 and PT5a); lane 4: PT14a (at least 10 copies of IS*200*), lanes 6–8 shows *Pvu*II–IS*200* patterns of the same strains. (b) *Sma*I-ribotype patterns. Lane 1: molecular size marker phage lambda *Hin*dIII; lanes 2 and 3: type III and type II according to [14] (strains P66040 and E2187 of PT3 and PT4; lane 4: PT9a (new type IX).

strains were shown to be of 5 IS*200* types when the RFLP-patterns of both *Pvu*II and *Pst*I were considered (Table 1). One IS*200* and one ribotyping pattern that had not previously been observed among *S*. Enteritidis strains are shown in Figure 1.

The strains of PT5a, PT9a, 11a, 20a, 31, 32 and 34 were of *Pvu*II and *Pst*I pattern I, and the strains are therefore included in *S*. Enteritidis clonal line I together with the type strains of PT1, 3, 4, 4a, 5, 6, 6a, 7, 9, 12, 17, 19, 20, 21 and 25. In support of this observation, Olsen and colleagues [14] typed clinical isolates of 20a, 31 and 32 and allocated these strains to the same clonal line, as has now been shown for the reference strains. The strains of PT13a and PT28 were allocated to clonal line II based on the presence of IS*200* type II with both enzymes. These phage types belong to the same clonal line as PT2, 8, 10, 13, 22, 23 and 24. The result with the PT13a type strain confirms the allocation of this phage type to clonal line II as previously suggested by a clinical PT13a isolate [14].

Olson and colleagues [14] observed that a wild-type strain of PT14b contained at least 7 copies of IS*200*. With the phage type reference strain, the *Pvu*II pattern differed from the pattern in the wild type strain, and the strain was found to contain at least 10 copies of the insertion element (Fig. 1). The strain of PT9b showed a pattern identical to one previously demonstrated in a clinical isolate of this phage type [14].

Based on previous studies [13, 14] and the results reported here, IS*200* typing is shown to divide strains of *S*. Enteritidis into two major clonal lines, each characterized by a unique IS*200* pattern. In addition, several minor groups are formed outside these two lines. Human cases may be caused by strains of all lines, but isolates from Europe have been predominantly of clonal line I, while clonal line II strains have been the most common on the American continent [1, 19]. There is, however, now a tendency towards a more complex picture, as for example PT4 outbreaks are reported from the USA [20].

LPS profiles

Thirty-seven of the 39 phage type reference strains possessed fully smooth LPS, while the strains representing phage type 7 and phage type 23 showed a shortened profile (data not shown) and were scored as semi-rough. The roughness of the strains was not evident from a simple visual examination of the colony morphology on agar plates. Strains of phage

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type 7 have been reported previously to be rough and of reduced virulence [21]. Phage type 7 strains may be derived from other phage types such as PT1, PT4 and PT6 under natural conditions, and such phage type conversions have been demonstrated apparently to be associated with loss of lipopolysaccharide [22]. Phage type conversion in *S.* Enteritidis has also been reported to be caused by introduction of extra chromosomal DNA [23, 24].

Mouse challenge experiments

LogVC10 values of the phage type reference strains of *S.* Enteritidis are shown in Table 1 in relation to the genomic line. Within the two major clonal lines, both strains of high (logVC10 > 7·5) and medium (7·5 > log VC10 > 5) to low (logVC10 < 5) virulence were present. The strains with high values had logVC10 values in the range 7·6–8·7 and included PT1, PT3, PT4, PT4a, PT5, PT9, PT17, PT19 and PT21 of clonal line I and PT2, PT8, PT13, PT22 and PT24 of clonal line II. The logVC10 values of these strains were not statistically different from each other. The strains of PT6, PT6a, PT12 and PT25 showed values of 5·1, 5·7, 7·0 and 7·2, while the two semi-rough strains, i.e. PT7 of clonal line I and PT23 of clonal line II, and the strain of PT10 from clonal line II were of low virulence, and logVC10 values of 4·6, 3·5 and 4·0, respectively, were obtained.

The mean logVC10 values of strains belonging to the three groups (clonal line I and II and other strains) were not statistically different, irrespective of whether or not the semi-rough strains were included in the calculations, indicating that IS*200* typing is not a useful method to distinguish between high and low virulence strains and that essential virulence factors are probably a common feature of all genomic lines of *S.* Enteritidis. The latter suggestion is supported by studies of population genetics within salmonella. The *spv* operon is believed to have been acquired early in *S. enterica* evolution, as the genes are present in the chromosome of strains belonging to subspecies II, IIIa and IV (and the Selander group VII), are carried on extrachromosomal elements of several of the more common serotypes of *S. enterica* subspecies I, but are not present in strains of *S. bongori* [24]. Other important genes for salmonella virulence have also most likely been acquired from an external source early in the evolution but after the split between salmonella and *E. coli*, i.e. genes belonging to the so-called pathogenicity islands (SPIs) [26]. The best characterized are SPI-1, which encodes genes involved in intestinal invasion and macrophage cytotoxicity [27] and SPI-2, which encodes genes required for survival within macrophages [28]. SPI-1 is present in all salmonellas while SPI-2 is absent in strains of *S. bongori* [26]. Thus it seems unlikely that high levels of virulence have been acquired separately by strains belonging to the different clonal lineages of *S.* Enteritidis, and it is a reasonable assumption that strains of moderate to low virulence in the mouse model have lost some of the essential factors needed to cause a systemic infection.

Ribotyping may be used as an alternative to IS*200* typing for determining genomic types of *S.* Enteritidis [14], and virulence was also assessed in relation to grouping determined by this method. Strains of PT3 and PT19 could be separated from the remaining strains of clonal line I that were tested in mice, based on a unique ribotype (Table 1). The type strains of these phage types both had logVC10 values in the high level. The type strain of PT10 (clonal line II) was of the same ribotype. The strain does not carry a virulence plasmid [29], and as expected, it was of low virulence. Strains of PT1, PT5, PT11, PT15 and PT20 are of one ribotype [14]. These strains were all shown to be highly virulent in the mouse model. However, the strain of PT6a showed the same ribotype, and this strain, although still in possession of its virulence plasmid, was only moderately virulent (Table 1). As with IS*200* typing, ribotyping was not a predictable indicator of virulence in *S.* Enteritidis.

In order to assess the variability of virulence within a phage type, more strains of PT1, PT2, PT4, PT8 and PT13 were tested (Table 2). All strains of PT1, PT2 and PT8 were highly virulent (logVC10 > 8·3) while medium to low virulent strains (logVC10 < 5·7) were observed among the PT4 and PT13 strains. This indicates that neither phage type, nor genomic typing is a predictable indicator of the virulence of *S.* Enteritidis strains. Strains of *S.* Enteritidis have been reported previously to vary in virulence, as have strains of the same phage type, and it has been noted that differences observed between strains in one animal model may not be detected in another animal model [7], indicating that different virulence factors are important in different animal species.

The reason for the variation in virulence observed in the present study is unknown, but may differ between strains. Mouse virulence drops dramatically when the virulence associated plasmid is removed [30, 31], but the demonstration of low virulent strains

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Table 3. *Adherence and invasion of strains of* S. *Enteritidis into MDCK cells*

Strain	Phage type	Clonal line	Adherence*	Invasion†
E2341	1	I	20±3	5±4
P66040	3	I	17±10	5±4
E2187	4	I	21±5	7±8
P99764	4a	I	25±6	4±5
P70001	5	I	20±5	9±3
P99327	6	I	13±1	11±10
E2408	6a	I	14±5	2±1
E1492	7	I	18±9	11±10
E2402	9	I	17±3	25±22
P95661	12	I	16±3	11±8
P95940	17	I	37±16	9±1
E1949	19	I	23±10	8±4
P72580	21	I	24±8	38±23
E2457	2	II	17±3	26±35
E2468	8	II	22±5	24±22
E3945	10	II	19±11	31±34
E464	13	II	20±4	7±0
P84357	22	II	18±6	13±10
P88255	23	II	61±4	4±1
P99768	24	II	28±9	151±34
E2109	11	Other	28±9	33±41
E2384	14	Other	22±9	11±3
E2400	15	Other	32±6	16±11
E866	16	Other	43±6	25±19
P89448	18	Other	40±18	106±6
P68147	20	Other	17±2	16±16

* Adhesion defined as associated bacteria as a per cent of inoculum.

† Invasion defined as intra cellular bacteria as a per cent of associated bacteria.

cannot be due simply to the absence of *spv* genes, as virulence plasmids and *spv*-genes have been demonstrated in all phage type reference strains tested, except PT10 [29]. environmental conditions often control expression of virulence factors in pathogenic bacteria [32], and PT4 strains that show a generally impaired ability to adapt to changing environmental condition have also been demonstrated to be essentially avirulent in mice by the oral infection route [10]. The strains have a variant *rpoS*-gene with a deletion, and in a direct comparison, a strain with the variant gene was less virulent in mice following oral challenge than a non-isogenic strain with the normal gene [33], while such strains do not differ in the ability to invade and colonize internal organs following oral challenge of one day old chickens [34]. Since the strains were not isogenic, however, other underlying factors may have caused the difference in virulence. Also, different sequence variations in the *E. coli katF* (*rpoS*)-gene have been shown to have different effects on the phenotype [35], and this may also be a possibility for similar mutations in strains of salmonella.

Strains originating from clinical cases seem to be more virulent than those from the environment [11], and it may be that strains of *S.* Enteritidis of low and medium virulence are common in nature but that animal and human hosts select for the more virulent variants. As the majority of studies dealing with virulence characterization have used clinical isolates this may give an unbalanced picture of the virulence variation with the serotype.

Adhesion to, and invasion of cultured cells

Results from *in vitro* adhesion and invasion assays with selected strains are summarized in Table 3. After 1 h of incubation, the number of c.f.u.s associated with the MDCK cells were highest for the semi-rough strain of 23. The per cent association of this strain was significantly higher than for all other strains except PT16, PT17 and PT18. This result is in accordance with previous observations that LPS-mutants adhere strongly to cells and other substances in *in vitro* assays [36]. Among the remaining strains, phage types 6 and 6a showed the lowest ability to adhere to cultured MDCK cells but the values observed were not statistically different from the values of other phage types, with the noted exception of phage type 23.

In an attempt to correlate virulence with an *in vitro* feature of strains of *S.* Enteritidis, Solano and colleagues [11] showed that when the bacteria were starved, only virulent strains were able to adhere to the wall of class tubes, forming a biofilm. No direct comparison with the present study was possible since different strains were tested and since bacteria in the present investigation were not starved before the ability to adhere to cells was tested.

After incubation with gentamicin for 2 h (intracellular bacteria only), 4% of the c.f.u.s associated with cells after 1 h could be detected in the positive control (*S.* Typhimurium 13/74), while the negative control showed 0% (*S.* Typhimurium invH$^-$) intracellular location. The values for most of *S.* Enteritidis strains varied between 5 and 40%. An apparent intracellular replication, however, was observed with PT24 (c.f.u. more than 150% of associated cells) and with PT18 (c.f.u. 106% of associated cells).

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In previous testing, cells of *S.* Enteritidis had been shown to be capable of adhering to, and entering cultured epithelial cells [37]. Based upon the present results it can be concluded that this ability seems to vary greatly between different strains of the serotype. However, due to the small number of tests performed with each strain, the standard deviation was high, and only a few strains differed significantly.

The tissue culture results did not correlate with the results of i.p. mouse virulence tests, possibly because the i.p. challenge model does not require invasion of the gut. However, for some of the strains included in the present study, studies of invasion in a chicken loop test model have been performed recently. In this model, PT4 was significantly better at invading than strains of other phage types (Aabo, personal communication). This was not reflected in the present *in vitro* adherence and invasion assays. Although it is desirable to reduce the use of experimental animal infections to a minimum, our results indicate that cell culture assays cannot be used to predict the outcome of the challenge of the mouse with *S.* Enteritidis.

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