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Virulence and genotype stability of *Salmonella enterica* serovar Berta during a natural outbreak

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SUMMARY

Strains of *Salmonella enterica* serotype Berta, collected over a period of 6 years from a well documented natural outbreak in Denmark, have been characterized in order to assess the stability of chromosomal typing systems and virulence properties. Outbreak strains were identical in *Pvu* II and *Pst* I IS*200* profiles, all but two strains showed the same *Sma* I ribotype, and all but one strain showed the same *Not* I pulsed field gel electrophoretic pattern, indicating that these molecular markers remained almost constant during the outbreak. In general, strains of *S.* Berta were found to be of moderate to low virulence; log VC_{10} values were found to vary between 3·0 and 4·4 after i.p. challenge of mice, and maximum CFU in internal organs of day-old chicks varied between 2 and 4 $\log_{10}$ units following oral challenge. The minor differences observed between strains *in vivo* did not correlate with differences in *in vitro* invasion into cultured MDCK cells, nor with *in vitro* growth characteristics. A succession of different plasmid profile types was observed during the outbreak but a hierarchical selection of clones based on differences in virulence was unlikely to have caused the succession of types of *S.* Berta during this outbreak.

INTRODUCTION

Salmonella enterica serotype Berta (Berta) (O:1,9,12; f,g,t:–) is mainly associated with poultry [1, 2] but has been reported in relation to large outbreaks of salmonellosis among humans, presumably due to consumption of poultry products [3, 4]. It is included among the salmonella serotypes that can be invasive in poultry [5] but it has not been reported to cause disease in these animals.

An outbreak caused by serotype Berta occurred in Denmark from 1984–92. This was the first recorded isolation of Berta in Denmark, and the outbreak was believed to be caused by a single source [1]. At the height of the outbreak more than 60% of all broiler flocks presented at slaughter were contaminated, and more than 10% of all reported cases of human salmonellosis were caused by Berta. This incidence ranked Berta as the third most common serotype from diseased humans from 1985–90. The infection was eradicated from broilers in 1991. Isolates were systematically collected and stored during the epidemic. The collection therefore constitutes a suitable source for studies into the behaviour of Berta during the epidemic.

We have previously reported on the plasmid content of strains of Berta obtained from the outbreak and shown that a clear succession in plasmid profile types occurred during the outbreak [1, 3]. In a search for more stable markers, we [6] and others [7], have further shown that geographically diverse strains of Berta showed surprisingly little chromosomal restriction fragment variation, and suggested that the serotype consists of a single clone of bacteria. Little is

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known, however, about the stability of chromosomal markers during long lasting outbreaks of *S. enterica*. In the present paper, stability of chromosomal markers and virulence characteristics of strains of serotype Berta obtained from the Danish outbreak are investigated.

MATERIAL AND METHODS

Bacterial strains

The origin and year of isolation of Berta strains used (Table 1) have previously been reported [1, 3, 6]; strains investigated originated from poultry, humans, cattle, pigs and slurry during the outbreak in Denmark. The collection also contained control strains obtained from poultry and humans in six other countries. Media and growth conditions used were as previously reported [3].

Typing methods

Ribotyping, using restriction enzyme *Sma* I, IS*200* typing using *Pvu* II and *Pst* I and pulsed field gel electrophoresis (PFGE), using restriction enzyme *Not* I, was performed as reported previously [8]. All restriction enzymes (Boehringer Mannheim or Amersham) were used as recommended by the supplier.

Mouse virulence

Mice (Balb/C females, 19–21 grams, groups of 10) were challenged intraperitoneally with 3×10^3 CFU from an overnight culture, and $\log_{10}$ of CFU per spleen 10 days post challenge ($\log_{10}$ VC10) was determined as reported elsewhere [9].

Challenge of day-old chicks

Day-old Brown Leghorn chicks were housed in cages in groups of 12. Water and feed were supplied *ad libitum*. Two birds from each group were sacrificed and tested for salmonella by the methods described below. The remaining birds were challenged *per os* with 5×10^6 CFU from an overnight broth culture. At days 2, 5, 8, 12 and 14, two birds from each group were sacrificed, and CFU/spleen, CFU/liver and CFU/yolk sac were determined by spread-plating 10-fold dilutions in PBS onto modified brilliant green agar (Oxoid CM329). Plates were incubated at 37 °C for 16 h. Caecal tonsils were incubated in selenite broth (Oxoid CM395) at 41·5 °C for 24 h. One loopful of this was spread on Rambach^R agar (Merck 7500-002) and incubated for 22 h at 37 °C. Standard procedures were used for identification of *S. enterica*, as previously reported [10].

Tissue culture assays

Adherence to and invasion into cultured MDCK (Madin Darby canine kidney) cells were determined by standard gentamicin assays [11]. Briefly, cells cultured in RPMI 1640 medium (Gibco-BRL) supplemented with 10% foetal calf serum and glutamine were used in all assays. Five $\times 10^5$ cells were added to each well of a 24-multiwell tissue culture plate (A/S Nunc, Roskilde, Denmark) and incubated overnight at 37 °C in a 5% CO_2, humidified atmosphere. Prior to the assay, the medium overlying the MDCK monolayer was removed and replaced with 1 ml of fresh, prewarmed RPMI medium. Bacteria were cultured at 37 °C in Luria-Bertani (LB) broth [12] to approximately 5×10^8 CFU/ml. Approximately 2×10^6 CFU, diluted in tissue culture media, were added to the monolayer. Each strain was assayed in triplicate. Cells were then incubated at 37 °C in a 5% CO_2 atmosphere for 1 h, after which the medium was removed and the cell monolayer was washed three times with prewarmed, sterile phosphate buffered saline. To each well was finally added 1 ml of fresh RPMI medium containing 100 μg/ml gentamicin (Gibco-BRL) and incubation was continued for a further 2 h. Cells were then washed three times with sterile phosphate buffered saline.

Adherence was expressed as $\log_{10}$ CFU/well after 1 h of incubation and invasion as $\log_{10}$ CFU/well after a further 2 h of incubation and with gentamicin present. The cell monolayers were lysed with 200 μl 1% Triton X-100 for 10 min to release the viable intracellular bacteria. After adding 800 μl of LB-broth and vigorously mixing the suspension with a pipette, dilutions were plated onto LB-agar plates to quantify the bacteria.

Growth kinetics

In vitro growth was characterized during growth in LB-media as follows: 1 ml of an overnight culture was inoculated into 100 ml of prewarmed (37 °C) LB-media and incubation was continued at 37 °C for 7 h,

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Table 1. *Bacterial strains and genotype of strains*

Berta (strain)	Source*	Typing results: IS*200* *Pvu* II	*Pst* I	Ribotype	PFGE
2700-84	Outbreak – poultry	I	I	I	I
161-85	Outbreak – poultry	I	I	I	I
3772-85	Outbreak – poultry	I	I	I	I
28009-85	Outbreak – poultry	I	I	I	I
433-88	Outbreak – poultry	I	I	I	I
3492-88	Outbreak – poultry	I	I	I	I
2231-89	Outbreak – poultry	I	I	I	I
41948-89	Outbreak – poultry	I	I	I	I
37857-86	Outbreak – humans	I	I	I	II
40735-87	Outbreak – humans	I	I	I	I
78798-87	Outbreak – humans	I	I	I	I
8847-88	Outbreak – humans	I	I	I	I
20053-88	Outbreak – humans	I	I	I	I
73-89	Outbreak – humans	I	I	I	I
17214-89	Outbreak – humans	I	I	IV	I
27029	Outbreak – slurry	I	I	I	I
ds004	Outbreak – cattle	I	I	I	I
DK1098	Outbreak – cattle	I	I	I	I
463	Outbreak – pig	I	I	I	I
1120	Outbreak – pig	I	I	IV	I
69K	Uruguay – pig	I	I	II	III
4485/84	Poland – poultry	I	I	II	III
1637/75	India – human	I	I	II	III
1063/72	India – poultry	I	I	II	III
8248/90	UK – poultry	I	I	I	I
6449/90	UK – poultry	I	I	I	I
5783/89	UK – poultry	I	I	III	IV
324744	Australia – human	I	I	I	I
321165	Australia – human	I	I	I	I
5895-90	USA – poultry	I	I	I	II
6051-90	USA – poultry	I	I	I	I
217537-90	USA – poultry	I	I	I	I
17921-90	USA – poultry	I	I	I	I
S. Typhimurium C5 (control in virulence exp.; virulent)†					
S. Typhimurium M206 (control in virulence exp.; avirulent)†					
S. Typhimurium 4/74 (control in tissue cell exp.; invasive)†					
S. Typhimurium 4/74-452 (control in tissue cell exp.; *invH*$^-$)†					

* Sources of strains of Berta have been described previously [1, 3, 7].

† These strains were not subjected to typing. Typhimurium strains 4/74 and 4/74-452 were kindly provided by Tim Wallis, AFRC Institute for Animal Health, Compton, UK.

at which time the stationary phase of the growth curve was reached in most strains. At 1 h intervals, samples were removed and OD_{450} was determined in a Labsystem multiscan Plus ELISA reader as the mean of four readings. The growth rate was expressed as the slope of the growth curve during the first 6 h. Strains included in this assay were: 2700-84, 3772-85, 3492-88, 41948-89, 433-88, 17214-89, 8847-88, 73-89, 37857-86, 40735-87, 78798-97 (see Table 1).

Statistics

Mean values of log VC_{10} were compared using Bonferronis pairwise comparison, and the computer software GraphPad InStat, version 2.04A (GraphPad Software). CFUs in the livers and spleens of chicks were considered statistically different if the means $\pm$ 1.96 S.D. were non-overlapping. Regression analysis of growth curves was performed using the

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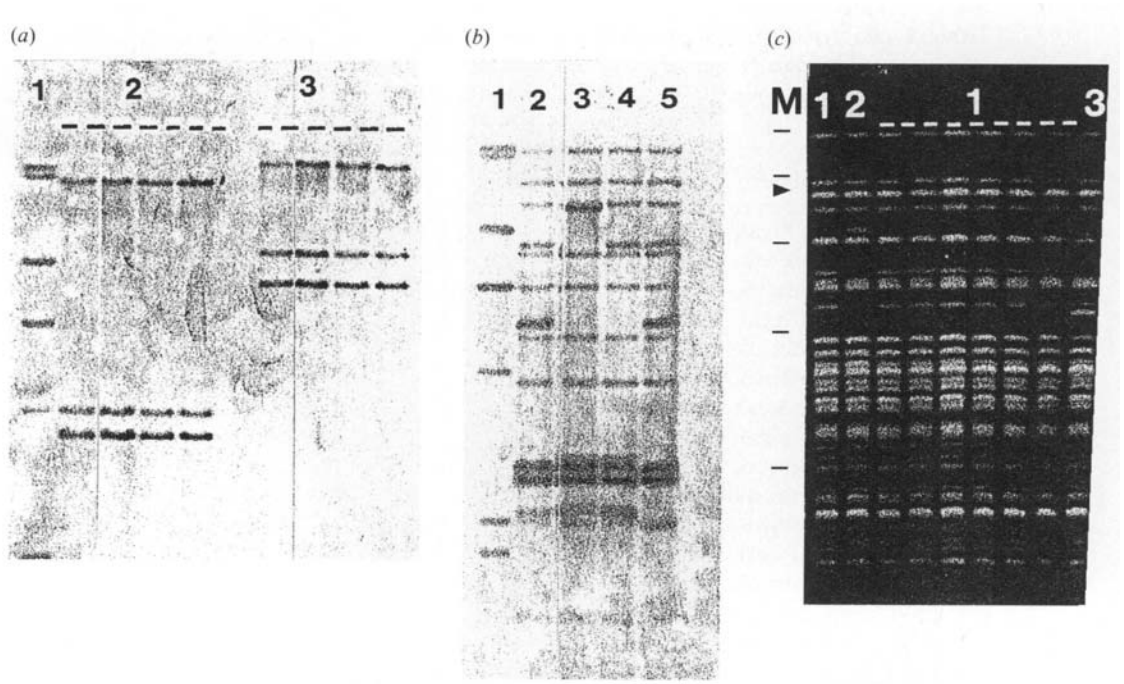

Fig. 1. IS*200* profile, ribotypes and PFGE patterns observed with strains of Berta. (*a*) IS*200* profiles. 1: Molecular weight marker, Lambda *Hin*d III; 2: *Pvu* II IS*200* profiles of four strains of Berta; 3: IS*200 Pst* I profiles of four strains of Berta. (*b*) Ribotypes. 1: Molecular weight marker, Lambda *Hin*d III; 2–5: Berta *Sma* I ribotypes I–IV (same four strains that show identical IS*200* profiles in (a)). (*c*) PFGE patterns. M: Molecular weight marker (Lambda multimers (48 kb)), 1 (7 strains) – 2–3: PFGE type I–III. PFGE type IV is not shown but strains with this profile type differ from type I only in the presence of one additional band in the position indicated by the arrow.

EPIINFO (CDC Atalanta, USA) software command regress.

RESULTS

Genotype variation of strains of *S.* Berta

The results of the molecular typing are listed in Table 1. All strains showed the same IS*200* profile, with three copies of IS*200* present. Four different *Sma* I ribotypes and four different *Not* I PFGE types were observed. All poultry and human isolates from the Danish outbreak were of the same ribotype except one isolate from a human and one isolate from a pig which showed a slightly different pattern. Likewise, one human isolate showed a PFGE type that was different from the remaining Danish isolates but identical to one isolate obtained from USA. In 28 out of 32 strains, grouping according to ribotype and PFGE type corresponded to each other. The restriction patterns observed with the three typing methods are shown in Figure 1.

Virulence of strains

Following i.p. challenge of mice, log VC_{10} values for strains of Berta varied between 3·0 and 4·4, compared to a log VC_{10} value of 8·5 for the fully virulent serotype Typhimurium C5 control strain and 2·0 for the avirulent M206 strain of serotype Typhimurium (Table 2). Three levels of log VC_{10} values were observed by pairwise comparison of means but the log VC_{10} value of 10 of the 12 strains tested were not significantly different from each other. The strain with the highest log VC_{10} value was isolated in 1985 and the strain with the lowest log VC_{10} in 1984. Early isolates of Berta in Denmark tended to be plasmid free whereas strains carrying 5·7 and/or 2·0 kb plasmids dominated late in the epidemic [1, 3]. The mean log VC_{10} values of the strains without plasmids were not significantly different from the strains carrying 5·7 and/or 2·0 kb plasmids.

The ability of strains to colonize and invade day-old chicks following oral challenge was also measured. Log_{10} CFUs obtained from spleen and liver post challenge are shown in Figure 2. All strains were reisolated from internal organs, but in low numbers; no strains reached a log_{10} CFU value greater than four per spleen or liver at any time during the 14 days where data were recorded. The differences in log_{10} CFU between different strains at the same day were not statistically different due to the low number of

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Table 2. *Mouse virulence of selected strains of Berta obtained during the Danish outbreak*

Strain	Plasmid profile	Source	Log VC_{10} χ^2	S.D.
2700-84	No plasmids	Poultry	$3{\cdot}3^{3}$	(0·1)
3772-85	No plasmids	Poultry	$4{\cdot}4^{1}$	(0·4)
78798-87	No plasmids	Human	$3{\cdot}8^{1,2,3}$	(0·2)
8847-88	2·0 kb	Human	$4{\cdot}0^{1,2}$	(0·8)
37857-86	2·0 kb	Human	$4{\cdot}2^{1,2}$	(0·2)
40735-87	2·0 kb	Human	$3{\cdot}8^{1,2,3}$	(0·8)
17214-89	2·0 kb	Human	$4{\cdot}3^{1,2}$	(0·2)
433-88	3·3 + 2·0 kb	Poultry	$3{\cdot}9^{1,2}$	(0·9)
3492-88	5·7 + 2·0 kb	Poultry	$4{\cdot}0^{1,2}$	(0·3)
41948-89	5·7 + 2·0 kb	Poultry	$4{\cdot}0^{1,2}$	(0·5)
73-89	5·7 + 2·0 kb	Human	$4{\cdot}2^{1,2}$	(0·3)
161-85	100 kb	Poultry	$3{\cdot}5^{2,3}$	(0·9)
S. Typhimurium C5			8·5	(—)
S. Typhimurium M206			2·0	(—)

* Mean log VC_{10} values were compared by Bonferroni's pairwise comparison of means. Three groups were identified (indicated in the upper case). More than one number with one strain indicates that the log VC^{10} of this strain can be grouped with two homology groups.

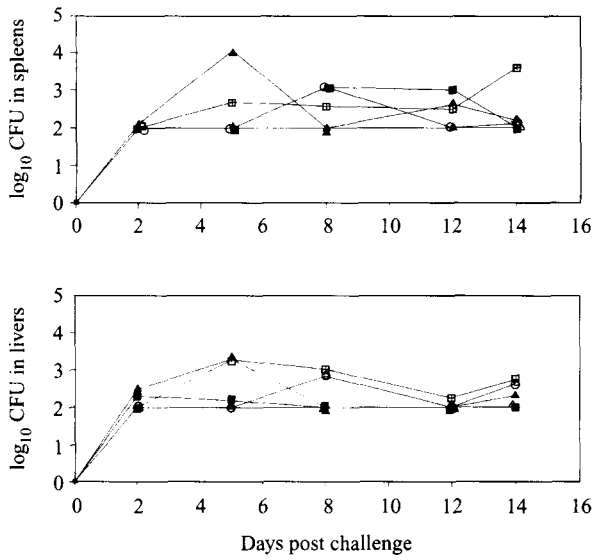

Fig. 2. Log_{10} CFU obtained from livers and spleens of day-old chicks challenged with strains of Berta. The strains used in these experiments were: △, 2700-84 (no plasmid), ○, 3772-85 (no plasmid), ■, 161-85 (100 kb plasmid), □, 8847-88 (2·0 kb plasmid) and ▲, 40735-87 (2·0 kb profile). The detection limit was 2 log^{10} CFU.

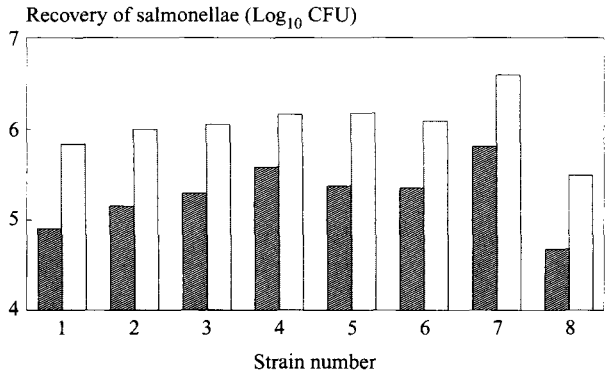

Fig. 3. The adhesion and invasion capabilities of strains of Berta into MDCK cell cultures. The average inoculum was 6·34 ± 0·12 log_{10} CFU/ml. The total associated bacteria, representing adherent organisms, were counted after 1 h of incubation. Intracellular bacteria were counted after a further 2 h of incubation in the presence of gentamicin. 1–6 are strains of Berta: JEO1234 (5·4, 3·3, 2·0 kb profile, first isolate obtained in Denmark), 2700-84 (no plasmids), 161-85 (100 kb plasmid), 3492-88 (5·7 + 2·0 kb plasmids), 433-88 (3·3 + 2·0 kb plasmids), 8847-88 (2·0 kb profile). 7–8: Tm/WT positive control (*S.* serotype Typhimurium) and Tm/*invH*⁻ negative control (*S.* Typhimurium).

birds per group. Bacteria were never recovered from yolk sac while caecal tonsils were positive in all birds at all times. No clinical symptoms were observed in any of the birds, and by macroscopic examination, no birds showed signs of pathological changes in internal organs.

A subset of strains were tested for their ability to adhere to and invade into cultured MDCK cells (Fig. 3). The proportion of Berta cells that adhered to MDCK cells varied between approximately 3 and 14 percent of the inoculum and was always lower than the highly adhering positive control, Typhimurium

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4/74 (32% adherence). Intracellular $\log_{10}$ CFU values of strains of Berta after 2 h of incubation with gentamicin present were intermediate between the non-invasive control, Typhimurium 4/74-452 and the positive control Typhimurium 4/74. Only the latter strain showed a net multiplication during the assay as compared to the $\log_{10}$ CFU in the inoculum.

In vitro growth characteristics

The slope of the growth curve during exponential growth at 37 °C varied between 0·066 (strain 17214-89) and 0·081 (strain 41948-89) OD_{450} units per hour. The mean for all strains was 0·073 units per hour and no strains showed a value which was significantly different from this mean.

DISCUSSION

Plasmid profiling has been shown to be the method with the highest discriminatory power for strain separation within serotype Berta [6], as has also been reported for other salmonella serotypes [13, 14]. However, conclusions based only on plasmid profiles may be uncertain. Plasmid profiles can change rapidly and have even been reported to evolve over such a short time period as five successive broiler flock generations [15]. Plasmids can also be lost during storage in stab cultures [16], and the same plasmid profile may be present in strains which are clearly different on a chromosomal level [8, 17]. Markers which are based on chromosomal DNA should therefore be used in parallel with a plasmid based typing system.

Three such chromosomal markers, IS*200* profile, ribotype, and PFGE-profile were shown to remain almost stable in strains obtained during a nationwide Danish outbreak of Berta. The only exceptions were three strains with differences in one of the methods ribotype or PFGE, respectively. It cannot be ruled out that the human isolate with a different PFGE type had been imported into the country via travelling, as detailed epidemiological data on this isolate are not available. The pattern produced was identical to a pattern observed in one poultry isolate from USA. The pig and human isolate with a different ribotype seemed to be part of the outbreak, and the small change in ribotype observed must therefore have evolved during the epidemic. The methods investigated are not suitable for epidemiological tracing of Berta during an outbreak due to the lack of discrimination, both between the outbreak strains and between these strains and the unrelated control strains.

IS*200* profiles were identical for all strains, which confirms results previously reported by Stanley and colleagues [7], except that they demonstrated one strain with a different profile. The particular profile is identical to the most common profile among strains of serotype Enteritidis. Their result may point to recombination taking place between Berta and Enteritidis, provided misclassification by serotyping can be ruled out. Compared to other common group-D salmonellae, strains of Berta seem unique in respect to copy number of IS*200*, as strains of the serotypes Gallinarum, Dublin and Enteritidis have been demonstrated normally to carry two copies of the insertion element IS*200* [7, 8, 18].

Four different *Sma* I ribotypes have previously been reported for Berta [6], but compared to the previous study, a new *Sma* I ribotype was present while one of the previously demonstrated types was absent in the present study. PFGE patterns have been reported to discriminate between strains that are otherwise identical both within serotype Enteritidis and serotype Gallinarum [8, 19]. Even with this method, however, Berta shows very little chromosomal variation. Based on the results of all three typing methods, it seems reasonable to conclude that this serotype is monophyletic.

Although documentation has not been presented to the knowledge of the authors, Berta is classified as invasive in poultry [5]. In accordance with this, bacteria were reisolated from internal organs but with $\log^{10}$ CFUs that are significantly lower than those reported in relation to the classical invasive serotypes. Such serotypes, which carry virulence plasmids [20], generally result in $\log_{10}$ CFU/spleen from 4–8 [21, 22] but following challenge with a dose 50 times higher than used here. Based on results with serotype Senftenberg, which causes only intestinal infection in adult chickens but may invade visceral organs in young chicks [23], it may be assumed that different salmonella serotypes are capable of invading day-old birds, and it still remains to be shown whether Berta can invade older chickens.

The experimental animals were observed for only 14 days. Most strains showed low $\log_{10}$ CFU values in internal organs at this stage but it is not possible from the present data to estimate how long Berta can

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persist in internal organs or in the enteric environment of chicks.

The strains tested demonstrated marginal variation in virulence towards both mice and chicks. There did not appear to be a general effect on virulence caused by long term storage of strains, as the second oldest strain exhibited the highest log VC_{10} towards mice. The infection kinetics in the chicks showed similarities to non-lethal infections caused by Gallinarum, Typhimurium and Infantis, in that approximately the same number of organisms were obtained from the liver and spleen, and the highest number of organisms were recovered at approximately 5 days [18, 24, Christensen JP, Olsen JE, Povlsen JS, Bisgaard M, personal observation].

Tissue culture assays are often used to evaluate the potential of salmonella strains to cause invasive disease [25–27], although the method's applicability has not yet been fully validated. According to the results of such assays, Berta has a medium to low capability for adhesion and invasion when compared with a fully virulent strain of Typhimurium; or once it has invaded, it has a low ability to survive. These two phenomena cannot be separated by the cell assay used. Mice were challenged intraperitoneally, and the minor differences in virulence suggested in the mouse model were therefore not associated with differences in degree of invasion in the gut environment. It could be possible that additional differences would have been observed if mice had been challenged orally. However, the strain's ability to adhere and invade in tissue culture assay were not statistically different, and approximately the same level of organisms were obtained in internal organs after oral challenge of chicks. Thus, strains of Berta seem to be uniform in their invasion phenotype. The minor differences in virulence could not be explained by differences in *in vitro* growth properties, either, as all strains seemed to grow at the same rate. Moreover, the duration of the lag phase and the plateau at which growth ceased were not different in the strains tested (data not shown).

In a further analysis of previously published data, where a clear succession of plasmid profiles was demonstrated during the Danish outbreak [1, 3], the apparent selection of one plasmid profile type over another was not found to correlate with invasion and growth in the liver and the spleen of chicks, cell culture invasion, persistence in experimental mouse infection or the improved ability to grow in artificial growth media. Most likely, natural selection for bacterial clones, in animals where antibiotic treatment is not used, is determined by many factors which interact in a complex fashion, and which cannot be revealed by simple virulence and growth assays.

In conclusion, all but three strains of Berta obtained during a natural outbreak showed the same patterns in genomic typing methods while minor virulence differences, which could not be correlated with succession of plasmid types during the outbreak, were observed. Berta is capable of invading day-old chicks following oral challenge and persisted in internal organs for at least 10 days following intraperitoneal challenge of mice. Berta was found to be of moderate to low virulence in both chicks and mice.

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