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

J. E. OLSEN*, M. N. SKOV, D. J. BROWN, J. P. CHRISTENSEN
AND M. BISGAARD

Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, 13 Bülowsvej,
DK-1870 Frederiksberg C, Denmark

(Accepted 11 January 1996)

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 IS200 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 VC₁₀ 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 log₁₀ 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

* Corresponding author and author to whom request for reprints should be addressed.

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, IS200 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% CO₂, 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% CO₂ 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,

Table 1. *Bacterial strains and genotype of strains*

Berta (strain)	Source*	Typing results			
		IS200 <i>Pvu</i> II	<i>Pst</i> 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
<i>S. Typhimurium</i> C5 (control in virulence exp.; virulent)†					
<i>S. Typhimurium</i> M206 (control in virulence exp.; avirulent)†					
<i>S. Typhimurium</i> 4/74 (control in tissue cell exp.; invasive)†					
<i>S. Typhimurium</i> 4/74-452 (control in tissue cell exp.; <i>invH</i> ⁻)†					

* 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 OD₄₅₀ 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 VC₁₀ were compared using Bonferroni's 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

Fig. 1. IS200 profile, ribotypes and PFGE patterns observed with strains of Berta. (a) IS200 profiles. 1: Molecular weight marker, Lambda *Hind* III; 2: *Pvu* II IS200 profiles of four strains of Berta; 3: IS200 *Pst* I profiles of four strains of Berta. (b) Ribotypes. 1: Molecular weight marker, Lambda *Hind* III; 2–5: Berta *Sma* I ribotypes I–IV (same four strains that show identical IS200 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 Atlanta, 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 IS200 profile, with three copies of IS200 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. Log₁₀ 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 log₁₀ CFU value greater than four per spleen or liver at any time during the 14 days where data were recorded. The differences in log₁₀ CFU between different strains at the same day were not statistically different due to the low number of

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	3.3 ³	(0.1)
3772-85	No plasmids	Poultry	4.4 ¹	(0.4)
78798-87	No plasmids	Human	3.8 ^{1,2,3}	(0.2)
8847-88	2.0 kb	Human	4.0 ^{1,2}	(0.8)
37857-86	2.0 kb	Human	4.2 ^{1,2}	(0.2)
40735-87	2.0 kb	Human	3.8 ^{1,2,3}	(0.8)
17214-89	2.0 kb	Human	4.3 ^{1,2}	(0.2)
433-88	3.3 + 2.0 kb	Poultry	3.9 ^{1,2}	(0.9)
3492-88	5.7 + 2.0 kb	Poultry	4.0 ^{1,2}	(0.3)
41948-89	5.7 + 2.0 kb	Poultry	4.0 ^{1,2}	(0.5)
73-89	5.7 + 2.0 kb	Human	4.2 ^{1,2}	(0.3)
161-85	100 kb	Poultry	3.5 ^{2,3}	(0.9)
<i>S. Typhimurium</i> C5			8.5	(—)
<i>S. Typhimurium</i> M206			2.0	(—)

* Mean log VC₁₀ 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 VC₁₀ of this strain can be grouped with two homology groups.

Fig. 2. Log₁₀ CFU obtained from livers and spleens of day-old chicks challenged with strains of *Berta*. The strains used in these experiments were: Δ , 2700-84 (no plasmid), $\circ$, 3772-85 (no plasmid), $\blacksquare$, 161-85 (100 kb plasmid), $\square$, 8847-88 (2.0 kb plasmid) and $\blacktriangle$, 40735-87 (2.0 kb profile). The detection limit was 2 log₁₀ CFU.

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.

Fig. 3. The adhesion and invasion capabilities of strains of *Berta* into MDCK cell cultures. The average inoculum was 6.34 ± 0.12 log₁₀ 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*).

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*.

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, IS200 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 investi-

gated 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.

IS200 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 IS200, as strains of the serotypes Gallinarum, Dublin and Enteritidis have been demonstrated normally to carry two copies of the insertion element IS200 [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

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 VC₁₀ 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.

ACKNOWLEDGEMENTS

This study was supported by the Danish Agriculture and Veterinary Research Council through grant No. 13-3548-1. The authors thank technicians Gitte Christensen, Tony Bønnelycke, Ole Aaslund and Charlotte Rasmussen for skillful assistance.

REFERENCES

1. Sørensen M, Brown DJ, Bisgaard M, Hansen HC, Olsen JE. Plasmid profiles of *Salmonella enterica* serovar Berta isolated from broilers in Denmark. *APMIS* 1991; **99**: 609–14.
2. Cox NA, Bailey JS, Mauldin JM, Blankenship LC, Wilson JL. Extent of salmonellae contamination in breeder hatcheries. *Poultry Sci* 1991; **70**: 416–8.
3. Olsen JE, Sørensen M, Brown DJ, Gaarslev K, Bisgaard M. Plasmid profiles as an epidemiological marker in *Salmonella enterica* serovar Berta infections. Comparison of isolates obtained from humans and poultry. *APMIS* 1992; **100**: 221–8.
4. Threlfall EJ, Hall MLM, Ward LR, Rowe B. Plasmid profiles demonstrate that an upsurge in *Salmonella berta* in humans in England and Wales is associated with imported poultry meat. *Eur J Epidemiol* 1992; **8**: 27–33.
5. Anonymous. Report of the Scientific Veterinary Committee on *Salmonella* serotypes to be considered invasive, and on sample based microbiological testing of poultry for slaughter for Finland, Norway and Sweden and updating the report for the Scientific Veterinary Committee of 11.11.1993 on alternative treatments when *Salmonella enteritidis* or *Salmonella typhimurium* has been confirmed in a poultry breeding flock. Brussels: Commission of the European Communities, 1994: 1–6.
6. Olsen JE, Brown DJ, Baggesen DL, Bisgaard M. Biochemical and molecular characterization of *Salmonella enterica* serovar Berta, and comparison of methods for typing. *Epidemiol Infect* 1992; **108**: 243–60.

7. Stanley J, Chowdry N, Powell N, Threlfall EJ. Chromosomal genotypes (evolutionary lines) of *Salmonella berta*. FEMS Microbiol Lett 1992; **95**: 247–52.
8. Olsen JE, Skov MN, Threlfall EJ, Brown DJ. Clonal lines of *Salmonella enterica* serotype Enteritidis documented by IS200, ribo, pulsed field gel electrophoresis and RFLP typing. J Med Microbiol 1994; **40**: 15–22.
9. Plant J, Glynn AA. Genetics of resistance to infections with *Salmonella typhimurium* in mice. J Infect Dis 1976; **133**: 72–8.
10. Aabo S, Andersen JK, Olsen JE. Detection of *Salmonella* in minced meat by the polymerase chain reaction method. Lett Applied Microbiol 1995; **21**: 180–2.
11. Fields PI, Swanson RV, Hardaris CG, Heffron F. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc Nat Acad Sci, USA 1986; **83**: 5189–93.
12. Maniatis T, Fritsch EF, Sambrooke J. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York, 1982.
13. Holmberg SD, Wachsmuth IK, Hickman-Brenner FW, Cohen ML. Comparison of plasmid profile analysis, phage typing and antimicrobial susceptibility testing in characterizing *Salmonella typhimurium* isolates from outbreaks. J Clin Microbiol 1984; **19**: 100–4.
14. Threlfall EJ, Hall MLM, Rowe B. *Salmonella gold-coast* from outbreaks of food-poisoning in the British Isles can be differentiated by plasmid profiles. J Hyg 1986; **97**: 115–22.
15. Brown DJ, Olsen JE, Bisgaard M. *Salmonella enterica*: infection, cross infection and persistence within the environment of a broiler parent stock unit in Denmark. Int J Med Microbiol 1992; **277**: 129–38.
16. Olsen JE, Brown DJ, Baggesen DL, Bisgaard M. Stability of plasmids in five strains of *Salmonella* maintained in stab cultures at different temperatures. J Appl Bacteriol 1994; **77**: 155–9.
17. Olsen JE, Skov M. Genomic lineage of *Salmonella enterica* serovar Dublin. Vet Microbiol 1994; **40**: 271–82.
18. Stanley J, Powell N, Jones C, Burnes AP. A framework for IS200, 16S rRNA gene and plasmid profile analysis in *Salmonella* serogroup D1. J Med Microbiol 1994; **41**: 112–9.
19. Christensen JP, Skov MN, Hinz KH, Bisgaard M. *Salmonella enterica* serovar Gallinarum biovar gallinarum in layers: epidemiological investigations of a recent outbreak in Denmark. Avian Path 1994; **23**: 489–501.
20. Gulig PA, Danbara H, Guiney DG, Lax A, Norel F, Rhen M. Molecular analysis of spv virulence genes of the salmonella virulence plasmids. Mol Microbiol 1993; **7**: 825–30.
21. Barrow PA, Simpson JM, Lovell MA, Binns MM. Contribution of *Salmonella gallinarum* large plasmid towards virulence of fowl typhoid. Infect Immun 1987; **55**: 388–92.
22. Barrow PA, Lovell MA. The association between a large molecular mass plasmid and virulence in a strain of *Salmonella pullorum*. J Gen Microbiol 1988; **134**: 2307–16.
23. Kodama H, Sato G, Mikami T. Age dependent resistance of chickens to salmonella *in vitro*: phagocytic and bacterial activities of splenic phagocytes. Amer J Vet Res 1976; **37**: 1091–4.
24. Barrow PA, Huggins MB, Lovell MA. Host specificity of *Salmonella* infection in chicken and mice is expressed *in vivo* primarily at the level of the reticuloendothelial system. Infect Immun 1994; **62**: 4602–10.
25. Barrow PA, Lovell MA. Invasion of Vero cells by *Salmonella* species. J Med Microbiol 1989; **28**: 59–67.
26. Galan JE, Curtiss III R. Distribution of the inv A, -B, -C, and -D genes of *Salmonella typhimurium* among other *Salmonella* serovars: inv A mutants of *Salmonella typhimurium* are deficient for entry into mammalian cells. Infect Immun 1991; **59**: 2901–8.
27. Khoramian-Falsafi T, Harayama S, Kutsukake K, Pechere JC. Effect of motility and chemotaxis on the invasion of *Salmonella typhimurium* into HeLa cells. Microb Pathogen 1990; **9**: 47–53.