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Biochemical and molecular characterization of *Salmonella enterica* serovar *berta*, and comparison of methods for typing

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SUMMARY

Strains of *Salmonella enterica* serovar *berta* (*S. berta*) from Denmark and seven other countries have been characterized with the aim of developing a rational typing strategy in connection with outbreak investigations.

Biotyping divided the strains into H_2S-positive (90%) and H_2S-negative (10%) biovars. Six percent of the strains were resistant to one or more antimicrobial agents. Eighty-eight percent of the strains carried plasmids and 52 different plasmid profiles were recognized. Six of the common plasmid sizes in these profiles were shown by restriction enzyme analyses to contain more than one plasmid species. More than 90% of the strains had the same ribotype with the restriction enzymes *Sma* I and *Eco*R I and the same whole cell protein profile. Outer membrane protein profiles and isoenzyme profiles were identical in all *S. berta* analysed.

Plasmid profiling in combination with restriction enzyme analysis of plasmids seemed to be the most rational typing strategy for *S. berta*. The results indicated that *S. berta* strains regardless of geographical source or host are possibly clonal in nature.

INTRODUCTION

Since the first reported isolation of *Salmonella enterica* serovar *berta* (*S. berta*) in 1936 from pigs in Uruguay [1], little attention has been paid to this serovar in most areas of the world. Bergey's Manual of Systematic Bacteriology [2] describes *S. berta* as a serovar with a limited geographic distribution (North America), and recent reports on isolation of *S. berta* have been concerned with broilers in the United States [3, 4].

S. berta was first isolated in Denmark in 1984 from imported broiler parent stock, and, subsequently, the number of broiler flocks infected with this serovar increased dramatically [5]. An almost concurrent epidemic among humans has been observed, and since 1985 *S. berta* has been the third most common serovar

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isolated from human cases of salmonellosis in Denmark (Gaarslev, personal communication). All epidemiological observations point to broilers as the main, if not the only source, for the epidemic among humans [6]. *S. berta* has also been reported from cases of human and animal salmonellosis in Australia and England/Wales [7, 8].

Detailed fingerprinting of bacterial isolates is an essential step in outbreak investigations [9, 10]. To develop a rational typing strategy for *S. berta*, a comparison was performed on biotyping, antibiotic resistance typing, plasmid profiling, plasmid restriction profiling, ribotyping, whole cell protein profiling, outer membrane protein profiling, and isoenzyme analysis of Danish isolates of *S. berta* obtained from the recent epidemics among broilers and humans. For comparison, the same analyses were performed on a reference collection of *S. berta* isolated from other sources or from other countries.

MATERIALS AND METHODS

Bacterial strains and media used for cultivation

A collection of 1284 *S. berta* strains isolated from poultry and humans from all parts of Denmark during 1984–9 has been established [5, 6]. A subset of this collection was selected to represent isolates with regard to geographic distribution, time of isolation, and plasmid profiles. These strains are listed in Table 1 together with a collection of *S. berta* strains isolated from other animal sources or isolated in countries other than Denmark. Strains were checked for purity and the serological diagnosis verified before the strains were characterized.

Subcultures were carried out on Tryptose blood agar base (Difco) and in LB-media [11] at 37 °C. Strains were stored at −80 °C in veal infusion broth containing 15% glycerol.

Biotyping

S. berta was characterized in 84 criteria as previously described [12, 13]. The 84 criteria are listed in Table 2.

Antibiotic resistance

Resistance to ampicillin, carbenicillin, cephalosporin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, neomycin, polymyxin B, streptomycin, sulfathiazole and trimethoprim, and tetracycline was determined using an agar disk method (Neo-sensitabs, Rosco) [14].

Conjugative transfer of R-plasmids was performed in broths by mixing 0·8 ml of the donor strain, 0·2 ml of the recipient strain *Escherichia coli* 14R525 (Nal^R) (courtesy of Dr E. J. Threlfall, Division of Enteric Pathogens, Central Public Health Laboratories, England), and 1·0 ml of fresh LB-broth. The mixtures were incubated overnight at 25 and 37 °C. 0·1 ml of the mating mixture was spread on McConkey agar plates (Oxoid CM109) containing 40 μg/ml nalidixic acid and one of the antibiotics to which the donor strain was resistant. Concentrations of antibiotics in the plates were 15 μg/ml for tetracycline and 20 μg/ml for all other antibiotics.

Table 1. *Strains of* S. berta *investigated*

Source	Country	Year of isolation	No.	Received from /strain number
Poultry	Denmark	1984–9	148	Ref. [5]
Humans	Denmark	1985–9	56	Ref. [6]
Cattle	Denmark	1987–9	3	B. B. Nielsen[a]
Pig	Denmark	1990	5	F. Bager[b]
Slurry	Denmark	1989	1	B. Munch[c]
Humans	Australia	1990	4	D. Lightfoot[d]
Poultry	USA	1990	12	R. M. Nervig[e]
Poultry	UK	1990	5	C. Wray[f]
Pig	Uruguay	1936	1	69K[g]
Human	Poland	1984	1	4485/84[g]
Poultry	India	1972, 1975	2	1063/72, 1637/75[g]

[a] B. B. Nielsen, National Veterinary Laboratory, Denmark.
[b] F. Bager, Federation of Danish Pig Producers and Slaughterhouses.
[c] B. Munch, Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Denmark.
[d] D. Lightfoot, Department of Microbiology, University of Melbourne, Australia.
[e] R. M. Nervig, National Veterinary Services Laboratory, Ames, Iowa, USA.
[f] C. Wray, Central Veterinary Laboratories, Weybridge, UK.
[g] Strains from the WHO Collaboration Centre for Reference and Research on *Salmonella*. Strains kindly provided by M. Y. Popoff.

Plasmid analysis. Isolation of plasmids for profiling was performed by the alkaline denaturation method described by Kado and Liu [15]. Plasmid DNA was separated in 0·8% agarose gels in a Tris acetic acid buffer [11] at 2·5 V/cm. Covalently closed circular (CCC) DNA was distinguished from open circular (OC) DNA by two-dimensional agarose gel electrophoresis [16].

Plasmid sizes were estimated from the migration in the agarose gels relative to the migration of reference plasmids in *E. coli* strains V517 and 39R861 [17, 18] by the method of Rochelle and colleagues [19]. Isolation of plasmid DNA for restriction enzyme analysis, digestion with restriction enzymes *Eco*R I and *Alu* I (Boehringer, Mannheim), and separation of restriction fragments in agarose gels were performed as previously described [20]. In addition to the digestion of the total plasmid content of strains, individual plasmids, of sizes less than 10 kb, were sliced from the agarose gels before digestion and purified from the agarose by use of a DNA-binding matrix (Prep-a-gene) as recommended by the supplier (Biorad). This purified DNA was then digested as described above.

Isolation of chromosomal DNA and ribotyping

Chromosomal DNA for restriction analysis was essentially isolated as described by Mekelanos [21]. Concentrations of DNA were estimated by spotting 5 μl samples, together with reference samples of herring sperm-DNA (Boehringer, Mannheim), on agarose plates containing ethidium bromide [22]. One μg of DNA was digested with restriction enzymes *Sma* I or *Eco*R I in potassium glutamate buffer [23] and DNA-fragments were subsequently separated in 0·8% agarose gels overnight at 30 V. Phage lambda (Boehringer, Mannheim) digested with restriction enzyme *Hin*d III served as a molecular size marker.

Table 2. *Biotyping of strains of* S. berta

Criteria	Reaction	%
Gram stain	−	100
Motile 22, 37 °C	+	100
Catalase	+	100
Oxidase, TMPD	−	100
Hugh and Leifson, glucose	fermentative	100
Phorphyrin test	+	100
β-haemolysin	−	100
Citrate, Simmons	+	100
Mucate, acid	+	100
Na-citrate, acid	+	100
Na-citrate, fermentation	+	100
D(−)tartrate, acid	+	100
D(−)tartrate, fermentation	+	100
Malonate, base	−	100
H_2S/TSI	d	90
Tetrathionate reductase	+	100
KCN, growth	−	100
Methyl red, 37 °C	+	100
Voges Proskauer, 37 °C	−	100
Nitrate reduction	+	100
Nitrate, gas	−	100
Urease	−	100
Alanin aminopept.	+	100
Arginin dehydrolase	+	100
Lysine decarboxylase	+	100
Ornithine decarboxylase	+	100
Phenylalaninedeaminase	−	100
Indole	−	100
Phosphatase	−	100
Gelatinase	−	100
Tween 20 hydrolase	+	100
Tween 80 hydrolase	−	100
McConkey, growth	+	100
Pigment	−	100
Glycerol, acid	d	81
Meso-erythritol, acid	+	100
Adinotol, acid	−	100
D(+)arabitol, acid	−	100
Xylitol, acid	−	100
D(−)arabinose, acid	d	85
L(+)arabinose, acid	+	100
D(−)ribose, acid	+	100
D(+)xylose, acid	+	100
L(−)xylose, acid	−	100
Dulcitol, acid	+	100
Meso-inositol, acid	−	100
D(−)mannitol, acid	+	100
D(−)sorbitol, acid	+	100
D(−)fructose, acid	+	100
D(+)fucose, acid	−	100
L(−)fucose, acid	−	100
D(+)galactose acid	+	100
D(+)glucose, acid	+	100
D(+)glucose, gas	+	100

D(+)manose, acid	+	100
L(+)rhamnose, acid	+	100
L(−)sorbose, acid	−	100
Cellobiose, acid	+	98
β-glucosidase	+	100
Lactose, acid	−	100
ONPG	−	100
Maltose, acid	+	98
D(+)melobiose, acid	+	100
Sucrose, acid	−	100
Trehalose, acid	+	100
D(+)melezitose, acid	−	100
Raffinose, acid	−	100
Dextrin, acid	d	16
D(+)glycogen, acid	−	100
Inulin, acid	−	100
Aesculin, acid	−	100
Aesculin, Fe	−	100
Amygdalin, acid	−	100
Arbutin, acid	−	100
Gentiobiose, acid	−	100
Salicin, acid	−	100
D(+)turanose, acid	−	100
α-fucosidase	−	100
α-galactosidase	+	100
α-glucosidase	+	100
β-glucoronidase	−	100
α-mannosidase	−	100
β-xylosidase	−	100
Methyl-α-glycopeptid.	−	100

+, > 90% positive; −, < 10% positive, d. > 10% but < 90% positive.

For ribotyping the DNA was transferred to nylon-hybridization membranes (Hybond-N, Amersham) by vacuum blotting as recommended by the supplier of the vacuum blotter (Phamacia, LKB). DNA was fixed to the membrane by baking at 80 °C for 30 min. A fragment of the ribosomal genes of *Legionella pneumophila* cloned in phage-lambda [lambda 142] [24] was labelled with digoxigenin-dUTP (Boehringer, Mannheim) by random priming [25]. Another probe derived from *E. coli* 16S and 23S rRNA (Sigma) was labelled by use of reverse transcriptase (Boehringer, Mannheim) as recommended by the supplier of the enzyme.

The probes were hybridized to membrane fixed DNA overnight at 65 °C in 5 × SSPE (0·75 M-NaCl, 0·01 M-NaH_2PO_4 pH 7·7, 0·01 M-EDTA), 0·5% blocking reagent (Boehringer, Mannheim), 0·1% *N*-lauroylsarcosine (Sigma), 0·02% sodium dodecyl sulphate (Merck). Post-hybridization washes were in 5 × SSPE, 0·2% SDS for 2 × 15 min at room temperature followed by 30 min at 56 °C. A digoxigenin-detection kit was used for detection of hybrids as recommended by the supplier (Boehringer, Mannheim).

Whole cell protein profiling

Samples for whole cell protein electrophoresis were prepared from 15 ml of an overnight broth culture by the method of Costas and colleagues [26]. The samples were profiled in discontinuous 12·5% SDS-polyacrylamide gels under electrophoretic conditions as described by Costas and colleagues [27].

Outer membrane protein profiling

Outer membrane proteins were isolated by the method of Achtmann and colleagues [28] and were electrophoresed under the same conditions as for the whole cell protein samples. *S. dublin* k2888 [29], *S. typhimurium* LT2, *S. enteritidis* E2949 [30], and *E. coli* HB101 served as controls.

Isoenzyme profiling

Thirty millilitres of an overnight broth culture were centrifuged at 6000 g for 10 min and the resultant cell pellet was washed in 10 ml of 10 mM-Tris, 1 mM-EDTA (pH 6·8). The pellet was then resuspended in 0·5 ml of 10 mM-Tris, 1 mM-EDTA, 0·5 mM-NADP (Sigma) (pH 6·8). The cell suspension was lysed by four freeze–thaw cycles (−80 °C to room temperature) and unbroken cells and cell debris were removed by centrifugation. The supernatant was aliquotted to sterile tubes and stored at −80 °C until use. The enzyme activity of the crude extract was examined following electrophoresis under non-denaturing conditions on 8% polyacrylamide gels run on a 2050 Midget Electrophoresis Unit (Pharmacia LKB) at a constant current of 25 mA per gel for 40 min. Enzyme preparations from the strains of *S. dublin*. *S enteritidis*, *S. typhimurium*, and *E. coli* mentioned under outer membrane protein analysis and *Pseudomonas alcaligenes* strain AB2281 (courtesy of Dr W. Frederiksen, State Serum Institute, Denmark) served as controls.

The samples were tested for the following enzymes: malic acid dehydrogenase, leucine dehydrogenase, fumerase, glucose-6-phosphate dehydrogenase, alpha naphthyl acetate esterase, alanine dehydrogenase, alcohol dehydrogenase, superoxide dismutase, aconitase and isocitrate dehydrogenase. Enzyme activity was detected as described by Shaw and Prasad [31].

RESULTS

Biotyping

Sixty-two strains (31 from poultry, 6 from humans and 6 from other sources in Denmark, and 19 foreign strains) were biotyped. As seen from Table 2, all strains examined showed identical reactions in 78 of the 84 criteria tested. Thirty-seven strains (44%) differed from the rest in one or more of the criterias: formation of acid from glycerol (19%), acid from D(−)arabinose (15%), acid from cellobiose (2%), acid from maltose (2%), acid from dextrin (16%), and lack of H_2S production in TSI medium (10%). Positive reactions in D(−)arabinose, glycerol, and cellubiose, were always late, while biotyping based on fermentation of dextrin was difficult to read. The H_2S-negative biovar was seen among poultry and human isolates from Denmark (5% of the strains tested), in the four reference strains supplied from the WHO Collaboration Centre for Reference and Research on *Salmonella*, and in one isolate from the USA.

Antibiotic resistance

As shown in Table 3, 175 strains were examined for antibiotic resistance. Eleven strains (6%) were resistant to one or more antibiotics, and one strain from the UK was resistant to three or more different antibiotics (multiple resistance). Antibiotic

Table 3. *Antibiotic resistance of* S. berta

Source and country	No. tested	No. (%) resistant	Pattern of resistance[a]
Poultry, Denmark	122	3 (2)	Tet (2)
			Amp, Car (1)[d]
Humans, Denmark	21	1 (5)	Tet (1)
Other, Denmark[b]	7	0 (0)	
Foreign countries[c]	25	7 (28)	Tet (1)
			Amp, Car[e] (3)
			Amp, Car, Tet[f] (2)
			Amp, Car, Kan, S+T[g] (1)

[a] Amp, ampicillin; Car, carbenicillin; Kan, kanamycin; S+T, sulpha+trimethoprim; Tet, tetracyclin. The number of strains with this pattern appears within parentheses.

[b] 3 isolates from pigs, 3 isolates from cattle, and 1 isolate from slurry.

[c] See Table 1 for details on the strains. One strain from Australia was resistant to tetracyclin, one strain from the UK was resistant to ampicillin, carbenicillin, kanamycin and sulph-trimethoprim. The rest of the resistant strains originated from United States.

[d] Resistance transferred by a 160 kb plasmid.

[e] Resistance transferred by 58 and 54 kb plasmids.

[f] Resistance transferred by a 54 kb plasmid.

[g] Resistance transferred by a 220 kb plasmid.

Table 4. *Plasmid profiles of* S. berta

Plasmid profile[a] (kb)				No. of isolates			
				Poultry-DK	Humans-DK	Other-DK[b]	Foreign[b]
100				17	5	—	—
100	42	5·7	2·0	5	—	—	—
100		5·7	2·0	5	—	—	—
100			2·0	5	—	—	—
	58			—	2	—	3
	42			4	3	—	—
	36			7	2	—	—
	29			3	5	—	—
		5·7		5	2	—	—
		5·7	3·3 2·0	6	3	1	—
		5·7	2·0	19	5	6	1
		5·4		6	3	—	—
			3·3	3	5	—	—
			3·3 2·0	5	—	—	—
			2·0	5	10	—	—
No plasmids				12	—	2	15
Other profiles[c]				41	11	—	6

[a] Profiles present in five or more isolates are shown in detail.

[b] The source of these strains are listed in Table 1.

[c] The number of other profiles was 23 among isolates from poultry-DK, 8 among isolates from humans-DK, and 5 among foreign isolates. The five profiles among foreign isolates were not seen among isolates from Denmark.

resistance was more common among foreign isolates than among Danish isolates ($P < 0{\cdot}001$ by Fisher's exact test).

Resistance was transferred by conjugation to *E. coli* in 6 of the 7 foreign strains. In each case the full resistance spectrum was transferred. Transferable resistance

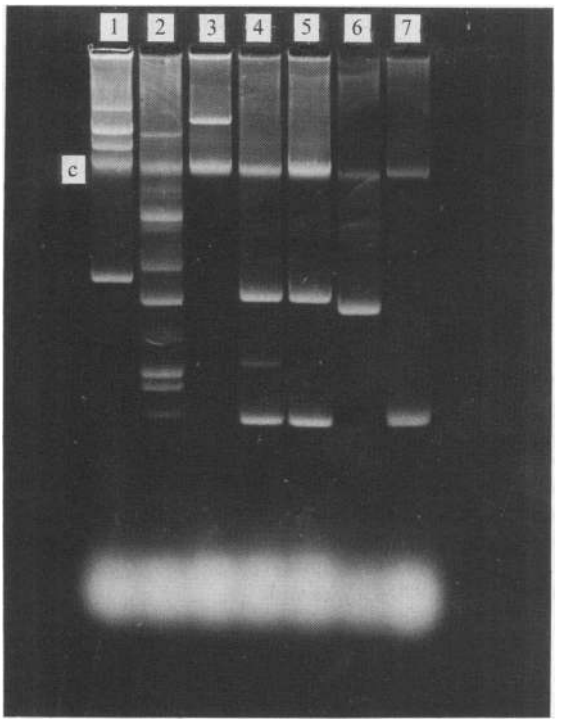

Fig. 1. The five most commonly demonstrated plasmid profiles among isolates of *S. berta*. Lanes 1 and 2, plasmid molecular size markers *E. coli* 39R861 (147, 63, 36 and 7 kb) and V517 (54, 7·2, 5·6, 4·4, 3·0, 2·7 and 2·1 kb). The three extra DNA bands between the chromosomal band (c) and the 7·2 kb band of V517, just above the 7 kb band of strain 39R861, represent open circular DNA. Lane 3, *S. berta* with the 100 kb profile; lane 4, *S. berta* with the 5·7, 3·3, 2·0 kb profile; lane 5, *S. berta* with the 5·7, 2·0 kb profile; lane 6, *S. berta* with the 5·4 kb profile; lane 7, *S. berta* with the 2·0 kb profile.

was only observed in one Danish isolate, where resistance to ampicillin and carbenicillin was transferred by a 160 kb plasmid.

Plasmid profiles

The plasmid content of all 238 strains are summarized in Table 4. Twenty-five different plasmid sizes and 52 different plasmid profiles were observed. Eleven profiles, comprising 52% of the poultry isolates and 83% of the human isolates, were common to Danish isolates from poultry and humans. All profiles demonstrated among Danish isolates from pig, cattle, and the environmental sources were also found among isolates from broilers. Three profiles (58 kb profile, 5·7, 2·0 kb profile, and the plasmid free profile), carried by 22% of the Danish isolates, were also demonstrated among foreign isolates. The five most commonly demonstrated profiles are shown in Fig. 1.

Plasmid restriction profiles

The nine plasmid sizes listed in Table 4 were analysed using restriction enzymes. The results are shown in Table 5. The 58 kb plasmids carried by strains from the

Table 5. *Restriction profile analysis of plasmids from* S. berta

Plasmid size (kb)	No. of plasmids analysed	Enzyme used	No. of profiles observed
100	23	*Eco*R I	5
58	7	*Eco*R I	2[a]
42	6	*Eco*R I	4
36	8	*Eco*R I	1
29	8	*Eco*R I	2
5·7	26	*Alu* I	2
5·4	11	*Alu* I	1
3·3	21	*Alu* I	1
2·0	23	*Alu* I	2

[a] One profile among Danish isolates and one profile among isolates from the USA.

Table 6. *Ribotypes of* S. berta

		No. with ribotype[a]							
Source of strain	No. analysed	E1[b]	E2[c]	E3	No. analysed	S1[d]	S2	S3	S4
Poultry, Denmark	30	28	1	1	27	26	1	—	—
Humans, Denmark	7	7	—	—	12	12	—	—	—
Other, Denmark[e]	4	4	—	—	5	5	—	—	—
Foreign strains[f]	16	16	—	—	16	12	—	3	1

[a] All strains were typed using the same probe. The type designation E1–E3 and S1–S4 refers to the use of the restriction enzymes *Eco*R I and *Sma* I, respectively, for digestion of the DNA.

[b] Three foreign strains with the E1 type had type S3 and one foreign strain had type S4. The rest of strains with the E1 had the type S1.

[c] One strain from poultry in Denmark had the E2 type. This strain correspondingly had the S2 type.

[d] One Danish strain from poultry with the S1 type had the type E3. The rest of the strains with the S1 type had type E1.

[e] Includes 2 isolates from pigs, 2 from cattle and 1 from slurry.

[f] Include 9 strains from the USA, 2 from the UK and India, and 1 strain from Australia, Poland, and Uruguay.

United States had restriction profiles differing from those obtained from plasmids of the same size in Danish isolates. The plasmids of the 5·7, 2·0 kb profile, demonstrated in one isolate from the UK, had the same *Alu* I profile as the Danish isolates carrying the same plasmids.

Ribotyping

Both of the enzymes *Sma* I and *Eco*R I produced several hundred restriction fragments in an agarose gel, and direct typing based on restriction fragment length polymorphism of chromosomal DNA was not attempted. Using a ribosomal probe derived from *L. pneumophilia*, four different ribotypes were demonstrated with *Sma* I and three different types with *Eco*R I. The distributions of types according to source of the strains are shown in Table 6. The most common ribotypes (*Sma* I type I and *Eco*R I type I) were demonstrated in 92 and 96% of

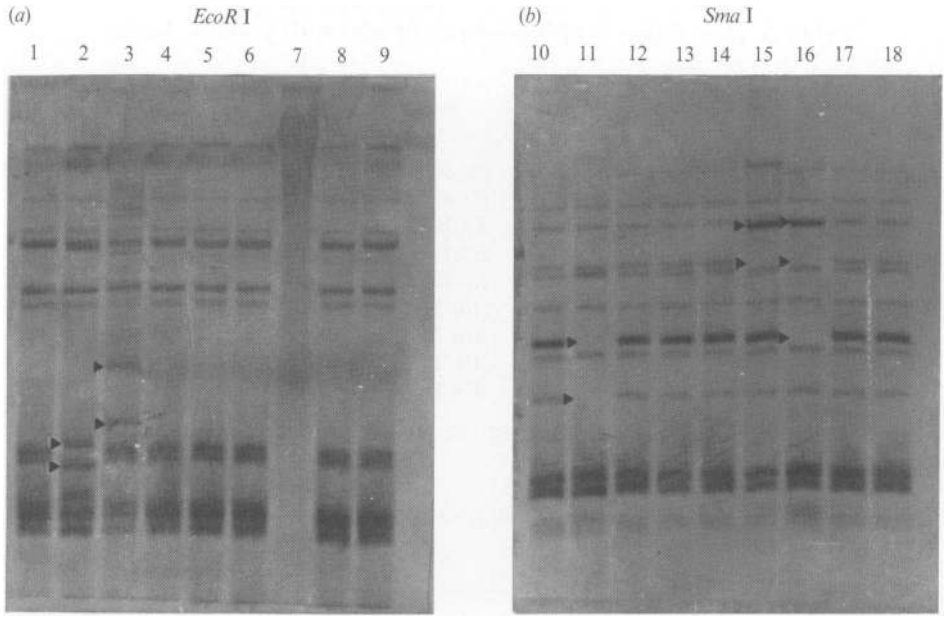

Fig. 2. Ribotypes of *S. berta* using an *E. coli*-derived rRNA probe. (*a*) Lanes 1–9 shows the ribotypes demonstrated using *Eco*R I for digestion of the DNA. Lane 1, *Eco*R I type I (Danish isolate from poultry); lane 2, *Eco*R I type II (Danish isolate from poultry); lane 3, *Eco*R I type III (Danish isolate from poultry); lanes 5, 6, 8, 9, *Eco*R I type I (Danish isolate from a human patient, an isolate from pig in Uruguay, an isolate from poultry in the UK, and an isolate from poultry in the USA), lane 7, empty. (*b*) Lanes 10–18 show the ribotypes demonstrated using *Sma* I for digestion of the DNA. Lane 10, *Sma* I type I (Danish isolate from poultry); lane 11, *Sma* I type II (Danish isolate from poultry); lanes 12–14, *Sma* I type I (Danish isolates from poultry, Danish isolate from a human patient, and an isolate from poultry in the UK); lane 15, *Sma* I type III (an isolate from pig in Uruguay); lane 16, *Sma* I type IV (an isolate from a human patient in Poland); lanes 17, 18, *Sma* I type I (an isolate from poultry in the USA and an isolate from cattle in Denmark). Arrows (▶) point to extra or missing bands in the ribotypes that differ from the most common types *Eco*R I type I and *Sma* I type I.

all strains examined. Fifty-two strains were analysed using both enzymes, and five ribotypes were produced when the two enzymes were used in parallel. One strain with a *Sma* I type-I pattern had a unique *Eco*R I type III pattern while four strains with the common *Eco*R I type I were the only ones to demonstrate *Sma* I types III and IV.

Representatives of each of the ribotypes were hybridized with a probe derived from *E. coli* rRNA. The same grouping and very similar, but not 100% identical patterns, were observed using this probe. The patterns using the *E. coli* probe are shown in Fig. 2.

Whole cell protein profiles

Among 58 strains examined (28 from poultry, 11 from humans, and 6 from pig, cattle and environment in Denmark, and 13 foreign strains) 55 (93%) had the same whole cell protein profile. As seen from Fig. 3, minor differences in the basic

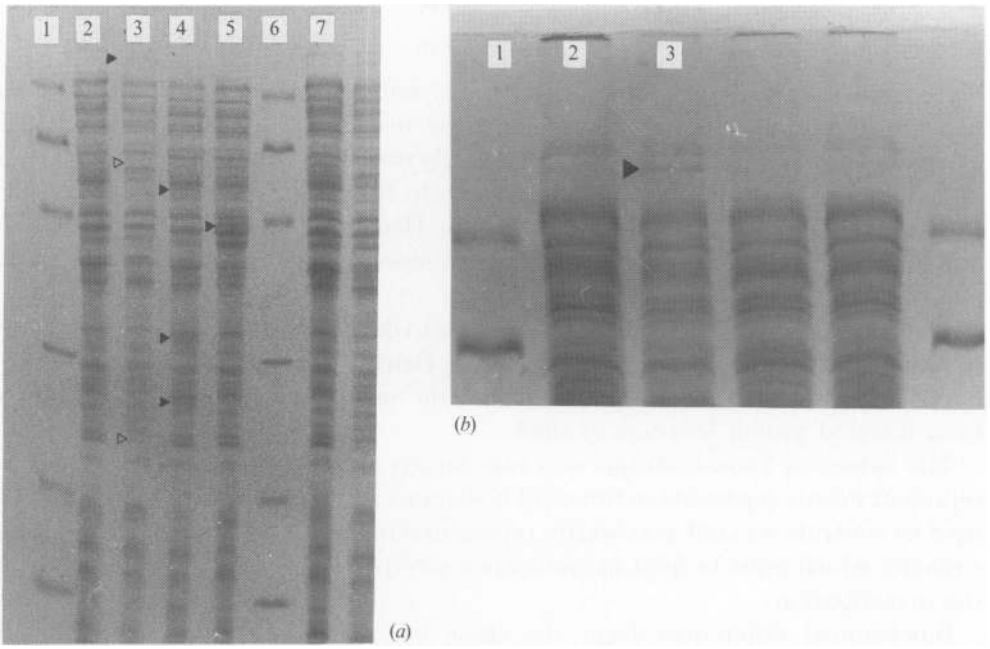

Fig. 3. (*a*) Whole cell SDS-protein patterns of *S. berta* obtained following discontinuous SDS–PAGE on a 12·5% polyacrylamide gel. Lane 1, molecular weight marker (97·4, 66·2, 42·7, 31·0, 21·5 and 14·4 kDa). Lane 2, *S. berta* strain from Danish poultry showing dominant protein profile. Lane 3, *S. berta* strain from Danish poultry demonstrating variant profile. Lane 4, *S. berta* strain from pig in Uruguay showing the second variant profile. Lane 5, Poultry strain from the USA showing the third variant profile. Lane 6, Same as lane 1. Variations in banding are indicated by arrows; closed arrow indicates an extra band and open arrow indicates where a band is missing compared to the dominant protein profile. (*b*) Close up of the top section of an overloaded gel with the protein samples of the same strains as shown in (*a*). The section is shown to highlight the additional high-molecular-weight band expressed by a strain from Danish poultry. The numbering refers to the same strains as in (*a*).

pattern were observed for one isolate from poultry in Denmark and the USA, respectively, and for one isolate from pigs in Uruguay. The differences did not correlate to plasmid content or to biotype.

Outer membrane protein profile

The 32 strains examined (16 from poultry, 9 from humans, 3 from cattle and 1 from a pig in Denmark, and 4 foreign isolates) exhibited the same outer membrane protein profiles. The patterns observed for *S. berta* were identical those of *S. dublin* and *S. enteritidis* strains used as controls, but were different from the outer membrane protein profile of *S. typhimurium*.

Isoenzyme analysis

The 53 *S. berta* strains examined (31 from poultry, 9 from humans, 6 from pigs, cattle and environment in Denmark, and 7 foreign strains), were monomorphic in all enzymes. The patterns observed for *S. berta* were identical to those of *S. dublin* and *S. enteritidis* strains used as controls, but different from the patterns of *S. typhimurium*, *E. coli*, and *Ps. alcaligenes*.

DISCUSSION

Since its introduction into Denmark with infected broiler breeders in 1984, *S. berta* has spread rapidly through all levels of the broiler production and in 1989 it accounted for more than 50% of all salmonella isolated from broilers [5]. There are no indications that reintroduction of *S. berta* to the country has taken place since 1984 [5]. Unlike other countries, *S. berta* in Denmark represents a considerable human health problem, and epidemiological observations point to broilers as the main source of human infections [6].

The present study was undertaken in order to develop a rational typing strategy to assist in the eradication of *S. berta* from Danish broilers, and to examine the hypothesis that all Danish isolates of *S. berta* are related to the strains isolated from infected broiler breeders in 1984.

The subset of Danish strains was not chosen at random, but was selected to represent events separated in time and in distance. The collection of foreign strains used as controls was not necessarily representative for *S. berta* in these countries, a matter which must be kept in mind when interpreting the results obtained from the investigation.

Biochemical differences form the basis of subdivision of *S. enterica* into subspecies [9]. For typing purposes, biotyping has the advantage that all strains are typable. Some authors consider it a less valuable typing method due to insufficiently proven stability [9] or lack of discriminatory power [32, 33]. It has, however, been a valuable typing method in the breaking down of several outbreaks of salmonellosis, either used alone or in combination with other typing methods [34, 35].

Biotyping of *S. berta* strains only revealed one fully reproducible variation (H_2S-production) which could be read after 24 h and which had a noteable frequency. The H_2S-negative biotype was recognized among Danish as well as foreign isolates, demonstrating a lack of discriminatory power for biotyping which is in agreement with previously published results [32, 33, 36]. All 62 strains met the biochemical description of *Salmonella enterica* subspecies *enterica* corresponding to Kaufmann group 1 [37, 38].

The use of antibiotic resistance patterns as a method for typing of isolates of salmonella has been shown to cause misleading results [32, 33]. One of the drawbacks concerning the method is that most resistance is carried on extrachromosomal elements, which are not always stable [9, 10]. The typability associated with the use of antibiograms as an epidemiological marker will, additionally, often be low as judged from reports on the general level of resistance among isolates of salmonella [39, 40]. Antibiotic resistance has been most valuable as a typing method within multiresistant clones, e.g. multiresistant *S. typhimurium* [41] and always in combination with other typing methods.

All *S. berta* isolates from Denmark, except 2%, were fully sensitive to the antibiotics tested. The two resistance profiles observed among Danish isolates were also demonstrated among foreign isolates, believed to be unrelated to the Danish strains.

The strains selected included all the different plasmid sizes recognized among Danish isolates of *S. berta* [5, 6]. A 160 kb plasmid which transferred resistance to

E. coli at 37 °C, was the only plasmid among Danish isolates to exhibit conjugative abilities. This plasmid has been demonstrated in only 2 of the 1284 Danish isolates from which the plasmid content has been reported [5, 6]. In contrast, R-plasmids were demonstrated in 21 % of the foreign strains just as resistance was significantly more common among the foreign strains.

Since plasmid profiling was introduced as a method for typing of bacteria [42], the method has been widely used in connection with outbreak investigations and in characterizations of salmonellae (e.g [43, 44]). Comparisons of methods for typing of isolates of salmonella, previously published, have mentioned plasmid profiling as a tool with high typability and a good discriminatory power [32, 33, 36]. Within *S. berta*, plasmid profiling has for example been used to compare isolates from humans with isolates from broilers [6].

Strains with no plasmids may be regarded as a type or they can be considered as 'non-typable strains'. In the latter case, the typability in the present study was 88 or 94 %, if only Danish isolates were included. Less than 1 % of Danish isolates from 1989 and onwards has, however, been without plasmids [5, 6]. The plasmid profiling divided the 238 strains into 52 groups, which was by far the best discrimination of the methods investigated.

The proportion of Danish strains carrying the different profiles were different from the proportions previously reported among poultry and human isolates [5, 6], possibly because the selection strategy was not random. The dominating profiles were, however, the same, and it was possible to compare the profiles of the Danish isolates to profiles of an unrelated 'control group' in order to investigate the apparent discriminatory power, i.e. the probability that unrelated strains will be ascribed to different groups [45].

Apart from strains with no plasmids (not typable), only two profiles (58 kb and 5·7, 2·0 kb) were shared among Danish strains and foreign strains, corresponding to a probability of 15 % that two unrelated strains would mistakenly be grouped together because of the same profile. The 5·7, 2·0 kb profile is the most common among Danish isolates [5, 6] and there may be an epidemiological relationship between the isolate from United Kingdom carrying this profile and Danish isolates. This assumption was supported by the identical restriction profile of the plasmids in the English and Danish strains. Taking this into account, the probability that a Danish isolate would mistakenly be related to a foreign isolate, because of the same profile, was 1 %. An apparent discriminatory power of 99 %, must, however, be evaluated by further studies dealing with epidemiological events that are more well-defined than the spread of *S. berta* in Denmark.

Thompson and colleagues [46], and later on Platt and colleagues [47], have recommended restriction enzyme analysis to ensure the identity of plasmids of the same size. The combined use of plasmid profiling and plasmid restriction analysis showed that only the 5·7, 2·0 kb profile was common to both Danish isolates and foreign isolates.

The restriction profile analysis showed that plasmid profiling used alone might result in misleading results, due to the fact that the common 100, 5·7 and 2·0 kb plasmids sizes were shown to comprise of more than one plasmid species.

The first isolate of *S. berta* from Denmark carried three plasmids (5·4, 3·3 and 2·0 kb) [5]. This strain was included in the present investigation, and it was

demonstrated that all three plasmids still exist among Danish isolates, although the profile itself has not spread successfully in Denmark. The 2·0 kb plasmids of the original isolate had a distinct restriction profile compared to the 2·0 kb plasmids now dominating in Danish strains, and the 5·7 kb plasmid, which appeared in 1985 and which in 1989 was carried by 97% of isolates from broilers [5] and 83% of isolates from humans [6] does not seem to have evolved from the original 5·4 kb plasmid.

Restriction fragment length polymorphism (RFLP) of chromosomal DNA was first used for typing of salmonella by Tompkins and colleagues [48] who demonstrated that most epidemiologically unrelated strains of *S. typhimurium* and *S. dublin* had unique hybridization patterns when hybridized with a radiolabelled random chromosomal probe. In the same year, it was suggested that ribosomal genes could be used as universal probes for the characterization of bacteria [49], and based upon this system, a sensitive typing method for *S. typhi* has been developed [50].

In the present study, a ribosomal DNA probe from a non-Enterobacteriaceae-species was used to characterize *S. berta*. A total of five ribotypes were observed when two restriction enzymes were used in parallel. For each of the enzymes, however, one of these profiles was carried by the majority of both Danish strains (98% *Sma* I and 95% *Eco*R I) and foreign strains (75% *Sma* I and 100% *Eco*R I), so although the typability was 100%, the discriminatory power was poor.

It is possible that additional types could have been demonstrated if other enzymes were included, but ribotyping seems, in line with the original suggestion of Grimont and Grimont [49] more informative in taxonomic studies than in outbreak investigations of salmonella infections. The uniformity of the patterns observed, points to a close relationship between all isolates of *S. berta*.

Whole cell protein profiles, like ribotyping, have mostly been used in taxonomic work, e.g. by Costas and colleagues [26, 27], but it has also proven to represent a valuable typing system, for example indicating clones of *Haemophilus parasuis* with certain virulence characteristics [51]. In the present study, whole cell protein profiles were evaluated as a typing method for *S. berta*, but like ribotyping, it was of no epidemiological value and only served to support the hypothesis that isolates of *S. berta* are closely related. All strains exhibited similar protein banding with a small number of extra bands in a few strains. The significance of the extra peptide bands is unknown and did not correlate to plasmid or biochemical differences between the strains. The observation may point to extra genetic information carried by certain strains, or they may reflect a different capability to express proteins.

Outer membrane proteins have been shown to differ among important *Salmonella* serovars just as single serovars may contain more than one profile [52]. In the present study, outer membrane profiling could not separate *S. berta* from *S. dublin* or *S. enteritidis*, and the method was considered of little value for epidemiological studies of *S. berta*.

Isoenzyme or multilocus enzyme (ME) typing has been an important method in population genetic studies of eucaryotes for many years. Although based on phenotypic expression of enzymes, it has been shown to detect more than 80% of all changes in the primary structure of the enzymes analysed [53]. Selander and

colleagues [54] proposed that the method could be used for population genetic studies of bacteria, and indeed such studies have been undertaken with promising results for a number of *Salmonella* serovars [55, 56]. As far as typing of strains in connection with outbreak investigations is concerned, ME has been used by Kapperud and colleagues [36] who found that strains of *S. typhimurium* within an outbreak could not be separated from unrelated strains by this method.

Our collection of *S. berta* all had identical ME type. The same ME type was observed in strains of *S. dublin* and *S. enteritidis* used as controls, an observation which limited the use of the method for typing purposes. The uniformity of the ME-types supported the hypothesis that strains of *S. berta* are closely related to each other, just as the results indicate that the three serovars *S. berta*, *S. dublin*, and *S. enteritidis*, all carrying the O-antigens 1, 9, 12, may in some way be evolutionary related to each other. The number of strains analysed is, however, far too small to elucidate this assumption.

In conclusion, plasmid profiling used in combination with restriction enzyme analysis of plasmids seems to be the most applicable typing strategy in epidemiological backtracing of *S. berta* infections. The time span where direct comparison of isolates can be performed by this method remains uncertain. If all Danish isolates, as assumed [5, 6] are descended from the clone(s) introduced in 1984, the build up of plasmid profiles within *S. berta* has been rapid, a matter which has to be taken into account when typing is performed.

In most situations, plasmid profiling is best used as a comparative method [9], i.e. typing must be performed in parallel, and there is a need for a more definite typing method for use in continuous surveys of *S. berta* infections among animals and humans. One such method could be phage typing which has formed the basis for continuous surveillance of infections with such prominent *Salmonella* serovars as *S. typhimurium* and *S. enteritidis* [30, 57].

Other typing methods such as biotyping, resistotyping, ribotyping, and SDS–PAGE profiling may be used for *S. berta*, and in some situations they may discriminate between isolates. Conclusions from such analyses must, however, only been drawn when strains have a different type, i.e. they are not related, while the demonstration of the same type does not necessarily indicate that strains are directly related, due to the very low discriminatory power for these methods within *S. berta*.

S. berta was shown to be almost clonal in nature based on both genotype (ribotype) and phenotypic expression of proteins (biotype, isoenzyme profiles, protein profiles). The Danish isolates were not distinct from the unrelated 'control group' in any of the markers supposed to be encoded by the chromosome (ribotype, isoenzyme profile, outer membrane protein profile, biotype, protein profile), and more than one ribotype was demonstrated in the group of Danish isolates. The few strains differing from the rest in ribotype could represent clones newly introduced into Denmark or they could indicate mutations in already introduced clones. Further studies are needed to clarify whether all Danish isolates are descended from the clone(s) introduced in 1984. The population of plasmids demonstrated among the Danish strains was unique, which would indicate that Danish *S. berta* have evolved for a period of time independent from the other *S. berta* analysed.

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