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Phage typing and PFGE pattern analysis as tools for epidemiological surveillance of *Salmonella enterica* serovar Bovismorbificans infections

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SUMMARY

Some years ago, an increase in the number of sporadic cases and outbreaks of salmonellosis due to *S. enterica* serovar Bovismorbificans was observed in several European countries including Finland, Sweden, England/Wales, Austria, and Germany. In order to understand the recent spread of this serovar and to trace the route of infection back to its source, it was considered necessary to subtype *S.* Bovismorbificans isolates. Using phage typing (newly described here) and molecular fingerprinting (PFGE-pattern, plasmid profiles and ribotype) the isolates of European origin could be subtyped and compared to *S.* Bovismorbificans isolates that originated in overseas countries such as Australia, Thailand, India, etc. where this serovar was isolated more frequently. Significant clonal diversity was identified but some of the clonal types of *S.* Bovismorbificans dominated the epidemics and single cases in Europe as well as in overseas countries. The clonal identity among these isolates indicates an international distribution, new sources of infection, and highlights the urgent requirement for standardized laboratory based surveillance networks (e.g. Enter-Net). Moreover, it is suggested that strains of *S.* Bovismorbificans will continue to be of concern in public health and that phage typing together with PFGE typing can be applied as reliable and rapid tools for their future monitoring.

INTRODUCTION

Salmonellosis is still one the most frequently recorded food-borne diseases, particularly in Northern countries [1]. Its causative agent, *Salmonella enterica*, appears in a great number of variants, such as serovars, electrotypes, genotypes phage types [2] etc. but strains of *S. enterica* sv. Enteritidis (*S.* Enteritidis) and of *S.* Typhimurium (*S.* Typhimurium) have been always ranking above the others. Strains of both serovars persist among animals of agricultural origin and in their vicinity, e.g. strains of *S.* Enteritis in poultry, particularly in eggs, *S.* Typhimurium strains among cattle and pigs, etc. Other *Salmonella* serovars, such as *S.* Agona, *S.* Virchow, *S.* Heidelberg, *S.* Derby, remain restricted in time and space and therefore, only of sporadic importance in human infections.

Beginning with the early nineties a re-emergence of human cases of salmonellosis due to isolates of sv. *S.* Bovismorbificans was recorded in several European countries [3–5], a salmonella sv. frequently observed

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in the 1950s but rarely after that [6, 7]. For instance, in Germany *S.* Bovismorbificans was ranking in the fifties and sixties among the top tens [6] but in the 1970s and 1980s with less than 0·1% among rarely occurring salmonellae. Between 1996 and 1998 this serovar was isolated from cases of salmonellosis as the third most frequently observed type with around 4% [5]. In contrast, in Australia this serovar can be found rather frequently among animal and human cases of gastroenteritis [8, 9]. It was suggested therefore that the emergence of salmonella strains belonging to sv. Bovismorbificans in European countries will be due to a particular pandemic bacterial clone. This might become comparable to the earlier observed pandemic spread of *S.* Enteritidis PT4 and *S.* Typhimurium DT104 infections ranking first in many European and American countries [10, 11].

In order to answer this hypothesis a collection of *S.* Bovismorbificans strains originated from Austria, Australia, Bahrain, Finland, Germany, Great Britain, Greece, Hungary, India, Sweden, Thailand and United Arab Emirates (UAE) were typed by molecular fingerprinting methods (PFGE, ribotyping, plasmid profiling) and by phage typing, using an as yet, unpublished system developed in the Australian Salmonella Reference Laboratory and published here comprehensively for the first time. The data presented in this paper reveal great clonal diversity of *Salmonella* sv. Bovismorbificans, even though more than 70% of all isolates in Europe belonged to a small number of clonal types. Moreover, phage typing together with PFGE fingerprinting were found reliable and rapid tools for laboratory based surveillance of *S.* Bovismorbificans.

MATERIAL AND METHODS

Bacterial strains

One hundred and sixty-two *S. enterica* sv. Bovismorbificans (*S.* Bovismorbificans) strains were included in this study (Table 1). They were selected from a number of *S.* Bovismorbificans strains sent to the National Reference Centres for Salmonellae in Finland, Australia and Germany for serotyping and other reference purposes. These strains originated from outbreaks and sporadic cases of gastro-enteritis among human beings as well as from foodstuff and from animals in Austria, Australia, Bahrain, Finland, Germany, Great Britain, Greece, Hungary, India, Sweden, Thailand and the United Arab Emirates (Table 1). However, overseas travelling was not reported among the Austrian, German or Finish patients. Additionally, three *S.* Bovismorbificans type culture strains were investigated for comparative purposes. All strains have been stored as glycerol (20%) cultures at −70 °C.

Phage typing

Phage typing was carried out as developed by C. Murray in 1982, and further completed by one of us (D. Davos). All phages were isolated from sewage effluent and propagated on respective propagation strains (see in Table 2, note 1) in the Institute of Medical and Veterinary Science (IMVS), the Australian Salmonella Reference Laboratory (ASLR). The medium for phage typing was Double Strength Nutrient Agar (D/S) agar (Difco Nutrient Broth D/S, sodium chloride 0·85%, agar 1·3%). Routine test dilutions (RTD) of each of the typing phages were loaded in special prepared wells in blocks and applied by a multipoint inoculator. From each fresh nutrient slope 5 ml nutrient broth was inoculated with a heavy inoculum and incubated on a shaker for 1·25 h at 37 °C. A bacterial lawn was immediately prepared on nutrient D/S agar plates using a Pasteur pipette (carefully removing all excess liquid from the plates). The plates were then inoculated with respective phages using a multipoint inoculator. The agar plates were incubated overnight at 37 °C after which the phage lysis could be read. The phage patterns (phage types) and the readings are summarized in Table 2. Some of the isolates gave rise to lysis reactions (sometimes weak) with the typing phages. However, the lysis patterns do not conform with the phage types defined in Table 2. Moreover, these atypical phage patterns were observed only with single isolates, respectively, and designated therefore operationally as RDNC (routine dilution, no conformity); they need further epidemiological evaluation.

Antibiotic susceptibility determination

Antibiotic susceptibility of the strains under investigations was determined as minimal inhibitory concentrations (MIC) to different antibiotics by the micro broth dilution test [12]. The MIC breakpoints of antibiotics for differentiation into susceptible (s) and resistant (r) strains were as follows (μg/ml): ampicillin s-1, r-16; cefotiam s-1, r-8; cefotaxime s-2, r-16; ceftazidime s-4, r-32; gentamicin s-1, r-8; kanamycin s-4, r-32; amikacin s-4, r-32; streptomycin s-8, r-64; chloramphenicol s-8, r-16; oxytetracycline s-1, r-8;

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Table 1. *Year of isolation, number and epidemiological origin of* S. *Bovismorbificans strains investigated throughout this study*

Year of isolation	Number of *S*. Bovismorbificans strains tested	Origin
1980	3	Reference strains, Germany
1993	3	Single cases of gastroenteritis in Germany
	1	Spice, Germany
	1	Calf, Germany
	2	Outbreak of gastroenteritis in Sweden
	1	Gastroenteritis, Finland
	1	Gastroenteritis, Indian
	1	Gastroenteritis, Greece
1994	6	Single cases of gastroenteritis in Germany
	1	Camel, Dubai, UAE
	5	Outbreak of gastroenteritis in Finland
	1	Gastroenteritis Thailand
	1	Gastroenteritis Bahrain
1995	3	Single cases of gastroenteritis, Germany
	1	Pork, Germany
	1	Chicken, Germany
	2	Camel, Dubai, UAE
1996	11	Single cases of gastroenteritis in Germany
	7	Diverse meat isolates, Germany
1997	15	Diffuse outbreak of gastroenteritis in Germany
	19	Single cases of gastroenteritis in Germany
	1	Compost isolate, Germany
	5	Various animals, Germany
	12	Different isolates from Australia
	3	Single cases of gastroenteritis, Austria
	3	Animals, Austria
1998	3	Diverse meat isolates, Germany
	15	Single cases of gastroenteritis, Germany
	8	Single cases of gastroenteritis, Australia
	15	Cattle, Australia
	1	Slaughterhouse, Australia
	1	Camel, Dubai, UAE
1999	4	Single cases of gastroenteritis Germany
	1	Chicken, Germany
	1	Chicken, Hungary
	1	Compost, isolate Germany
	1	Meat isolate Germany
	1	Gastroenteritis, Great Britain

ciprofloxacin s-1, r-4; sulfamerazin s-32, r-256; trimethoprim/sulfamerazin s-4, r-32.

DNA isolation, PCR, and plasmid profile determination

Chromosomal *S*. Bovismorbificans DNA isolation, DNA cleavage with restriction enzymes, and agarose gel electrophoresis were performed according to Ausubel et al. [13]. Elution of DNA fragments from agarose gels was carried out with GFX™ PCR kit (Pharmacia-Biotech, Germany). A PCR for the identification of plasmid fimbrial gene *pef* was carried out according to Bäumler et al. [14]. Plasmid DNA of *S*. Bovismorbificans strains was extracted by the method of Kado and Liu (15). The DNA probes used for ribotyping and were generated essentially as described by Prager et al. [11].

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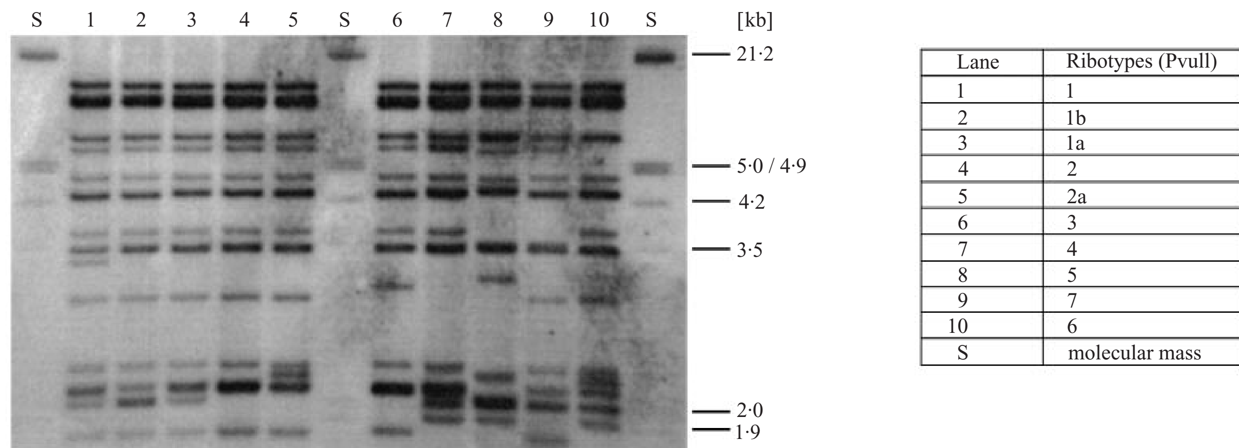
Table 2. *Phage typing scheme of* S. *Bovismorbificans*

Phage type	Phages 1	2	3	4	5	6	7	8	9	10
1	—	—	++, l	—	—	—	CL	SCL	—	—
2	—	—	—	—	—	—	—	OL	—	—
3	—	—	—	—	—	—	CL	CL	OL, +++ s	—
4	—	—	—	—	—	—	CL	CL	—	—
5	—	—	—	—	—	+++	CL	CL	CL	
6	—	—	± s,	OL	+++ s	SCL, + s	—	CL	CL	+ s
7	—	—	± n, s	OL	< OL	CL, + n	CL	CL	OL	< OL, + n, s
8	< OL	OL	OL	OL	OL	OL	CL	OL	+ n	OL
9	—	—	± n,s	OL	< OL	CL, + n	CL	CL	+ n	< OL
10	—	—	CL	—	CL	CL	CL, +++ s	± n, s	± n, s	SCL
11	+ s	+++ s	+++ s	CL, +++ s	OL, +++ s	CL	CL	OL	CL	+++ s
12	++ s	+++ s, m	+++ s	+++ m	+++ s	CL	—	CL	CL	+++ s
13	OL	OL	CL	CL	CL	CL	CL	CL	CL	CL
14	< OL	OL	OL	CL	CL	CL	—	CL	OL	CL
15	—	SCL	OL	OL	CL	CL	CL	CL	CL	CL
16	+++ s	CL	CL	< CL	CL	CL	CL	CL	SCL	CL
17	—	—	± n	± n, s	< OL	SCL	CL	CL	OL	CL
18	—	—	—	+++ s	—	+++ s	++ s	CL	CL	—
19	—	—	—	—	—	—	++ s	CL	+++ s	—
20	—	++ s	< OL	SCL	++ s	< CL	++ s	CL	CL	+ s
21	—	—	—	+++ s	++ s	+++ s	CL	CL	SCL	—
22	—	—	—	—	—	+++ s, m	—	CL	+++ s, m	—
23	—	—	—	—	+++ s	—	+++ s	CL	—	—
24	—	—	—	—	—	—	CL	—	—	—
25	—	OL	+++ s, m	+++ s	OL	< CL	CL	CL	< CL	++ s
26	—	—	± n, s	± s	OL	CL	CL	OL	± s	± n, s
27	—	< OL	—	+ s	< OL	—	+++ s	OL	—	—
28	—	2 n	OL	± s	CL	CL	CL	OL	+ n, s	OL
29	—	CL	± s	± s	CL	< CL	CL	CL, ++ s	—	++ s
30	—	—	—	± m	± m	± m	—	+++ s	+ m	—
31	—	SCL, +++ s	—	—	< OL	—	—	—	—	—
32	—	—	—	+++	+	—	SCL ±	SCL	SCL	—
33	—	—	+ m	—	+ m	—	+ m	—	+++	—
34	—	—	—	—	SCL	SCL	—	+ s	++ s	+++ s
35	+ s	+++ s	—	—	++ s	+++ s	—	+ s	—	—
36	—	—	—	+ m	± m	—	CL	—	< SCL	—

* The propagation strains for the typing phages are the following: 1, BM12; 2, BM20; 3, BM13; 4, BM15; 5, BM13; 6, BM11; 7, BM14; 8, BM16; 9, BM9; 10, BM19.
† CL, clear lysis; < CL, less than clear lysis; OL, opaque lysis; < OL, less than opaque lysis; SCL, semiconfluent lysis; < SCL, less than semiconfluent; +++, 80–100 plaques; ++, 40–80 plaques; +, 20–40 plaques; ±, 5–20 plaques; l, large; n, normal; s, small; m, minute; μ, micro.



Lane	Ribotypes (Pvull)
1	1
2	1b
3	1a
4	2
5	2a
6	3
7	4
8	5
9	7
10	6
S	molecular mass

Fig. 1. Ribotypes identified among *S.* Bovismorbificans.

Ribotyping

DNA isolation, restriction digestion, agarose gel electrophoresis, blotting, hybridization and detection of hybrids were essentially carried out as described earlier [11]. For ribotyping the restriction enzyme *Pvu*II was applied. Digoxigenin-labelled phage lambda-DNA digested with *Eco*RI and *Hin*dIII (Roche Diagnostics, Germany) served as molecular mass marker. The designation of ribotypes was made arbitrarily by numbering, the most frequent type was given number 1, then 2, etc. (Fig. 1). In addition to these numbers the letters (a, b, c) are used to designate closely related patterns (as seen from Fig. 1).

Pulsed field gel electrophoresis (PFGE)

Genomic DNA embedded in agarose was prepared as previously described [16] with minor modifications. The restriction enzyme *Xba*I, was applied according to the purchaser's recommendations: Chromosomal DNA of *S.* Typhimurium LT2 digested with *Xba*I was used as molecular size marker [17]. Pulsed field gel electrophoresis (PFGE) was carried out on CHEF DRIII (Bio–Rad, Munich, Germany) at 14 °C in 1·2% agarose gels for 40 h with a constant voltage of 200 V. Pulse times were 15–40 sec during the first 10 h, 30–50 sec for the following 22 h and 50–90 sec for the last 8 h. The reading and interpretation of PFGE pattern was carried out according to Claus et al. [18], using the RFLPScan™ System (Scananalytics, USA). The designation of PFGE pattern was made arbitrarily by numbering, e.g. the most frequent type: number 1; then the next most frequently observed pattern: number 2, if differences of patterns refer to more than four fragments. In addition to the numbers the letters (a, b, c) were used to designate closely related patterns (differing only in 1–2 fragments, see Figs 2, 3).

Biochemicals, enzymes, reagents

Restriction enzymes, DNA labelling and detection kits were purchased from Roche Diagnostics Germany and the PCR reagents from Applied Biosystems, Germany, respectively.

Designation of clonal types

Clonal types have been defined arbitrarily by numbers on the basis of PFGE patterns and phage types as described earlier [11], e.g. clonal type 1-20 means that a distinct PFGE pattern designated arbitrarily as 1 and a distinct phage type designated 20 was associated; a clonal type 1-14 means that PFGE pattern 1 appeared now with an other phage type namely 14, clonal type 2-14, means a different PFGE pattern designated 2 is associated with phage type 14 etc. (see Table 3).

RESULTS

Subtyping of *S. enterica* sv. Bovismorbificans isolates

Unusually large numbers of *S. enterica* serovar Bovismorbificans (O: 6,8 H:r: 1,5) isolates were detected during surveillance of human salmonellosis in various countries. A collection of 162 of such strains were subtyped by antibiogram, phage typing,

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Table 3. *Clonal types and properties of* S. *Bovismorbificans isolates*

Clonal type	Year of isolation	Number of isolates tested	Geographical origin	PT	Ribotype	PFGE
1-12	1996–9	12	Germany, Australia	12	1	1a, b, d
1-14	1993–9	55	Germany (diffuse outbreak), Austria, Greece, Australia, Hungary	14	1	1a, b, d
1-20	1996–7	23	Germany	20	1	1a, c
1-22	1995–6	6	Germany, Austria	22	1	1a, b, 1c, 1f
1-32	1994, 1998	19	Finland (outbreak), Australia, Germany	32	1	1a, b, e, g, h
1-ut	1997–8	13	Australia, Germany	UT	1	1a,b,h
1-1	1995	1	Germany	1	1	1
2-nz	1980	1	Germany	RDNC	2a	2
3-22	1980	1	Germany	22	2	3
4-ut	1994	1	Germany	UT	1	4
5-nt	1996	1	Germany	RDNC	5	5
6-14	1997	1	Germany	14	1	6
7-20	1997	3	Germany	20	1	7
7-ut	1998	1	Australia	UT	1b	7
8-nt	1997	1	Germany	RDNC	3	8
9-12	1993	2	Sweden	12	1	9
10-nt	1993	1	India	RDNC	2	10
11-12	1993	1	Finland	12	1	11
12-12	1994	1	Thailand	12	1b	12
13-14	1994	1	Bahrain	14	1	13
14-12	1994	1	Finland	12	2	14
15-nt	1994	1	UAE	RDNC	4	15
16-nt	1995	2	UAE	RDNC	4	16
17-ut	Reference	1	Australia	UT	1	17
17-16	Reference	1	Australia	16	1	17
18-14	Reference	1	Australia	14	1	18
19-ut	Reference	1	Australia	UT	1	19
20-nt	Reference	1	Australia	RDNC	1	20
21-31	Reference	1	Australia	31	1	21
22-ut	1998	1	UAE	UT/O1	6	22
23-ut	1999	1	Germany	UT/O1	7	23
24-ut	1998	1	Australia	UT/O1	1b	24
25-14	1998	1	Australia	14	1b	25
26-ut	1998	1	Australia	UT/O1	1	26
27-14	1998	1	Australia	14	1b	27
28-32	1998	1	Australia	32	1b	28

For abbreviations used see Table 1.

ribotyping and PFGE typing in order to describe their epidemiological relatedness.

Phage types

A broad spectrum of 36 phage types (PT) for *S.* Bovismorbificans could be established (Table 2); however, among the epidemic strains in Europe only five phage types (PT12, PT14, PT20, PT22 and PT32) predominated (Tables 3, 4). Some of the strains were untypable (UT) by the phage typing set but some showed reaction with the salmonella O1 phage (UT/O1). The different readings of PT12 and PT14 due to a weak reactions of the phages 1–5 for PT12 have been found easily and reproducible to carry out. The stability of the phage types has been scored when outbreaks strains remain of identical phage type after several subcultivation and storage in the type culture collection.

Antibiogram

Many of the isolates were found to be sensitive to antibiotics and only some of them resistant to sul-

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Table 4. Genetic variation noted among main clonal types

Clonal Type	PFGE	Plasmid profiles (in Md)	Antibiotic resistance	Year of isolation	Number of isolates	Orgin
1-12	1a	—	—	1996, 1997	3	Germany
		3·0	—	1997	1	Germany
	1b	60*	—	1997	2	Germany
		60*, 3·0	—	1998	1	Australia
		4·6	SuTp	1996–1999	4	Germany
	1d	60	SSu	1997	1	Germany
1-14	1a	—	—	1994, 1996, 1999	7	Germany
		3·5, 3·0	—	1995	1	Germany
		6·0	SSu	1996	1	Germany
		—	SSuT	1996	1	Germany
		3·0	—	1997	2	Germany
		3·0	ST	1997	1	Germany
		60*	—	1999	2	Hungary, Germany
	1b	—	—	1993–1999	17	Austria
		—	SSu	1997, 1998	3	Germany
		3·0	—	1993	1	Germany
		3·0, 1·8	—	1993	2	Germany
		1·8	—	1996	1	Germany
		3·5	—	1997	1	Germany
		3·5, 3·0	—	1997	1	Germany
		4·6	SuTp	1997	1	Germany
		60*	—	1993, 1994, 1998	7	Australia
		60*, 3·0	—	1998	1	Australia
		60*, 3·0	S	1998	1	Australia
		60*, 3·5	—	1997	1	Germany
		60*, 1·8	—	1997	1	Austria
		60, 4·6, 3·0, 1·8	SSuTp	1998	1	Germany
	1d	—	—	1996	1	Germany
1-20	1a	—	—	1996, 1997	15	Germany
		100	—	1996	1	Germany
		60	—	1996	1	Germany
	1c	—	—	1996, 1997	5	Germany
		—	ACSu	1996	1	Germany
1-22	1a	—	—	1995, 1996	3	Germany
	1b	3·0	—	1996	1	Germany
		60*, 2·2	—	1996	1	Germany
	1f	60*	—	1980	1	Reference
1-32	1b	60*	—	—	3	Australia
		60*, 3·0	—	1998	8	Australia
		60*, 3·5, 3·0	—	1997	2	Australia
	1e	60	—	1997, 1998	2	Germany
	1g	60*	—	1994	4	Finland
1-ut	1a	—	A	1998	5	Germany
	1a	30	A	1998	4	Germany
	1b	—	SSu	1997	1	Austria
		60*, 3·5	—	1997	1	Germany
	1h	60*, 38, 3·5, 3·0	KT	1997	1	Australia
		60*	—	1997	1	Australia

* pef-positiv, serovar-specific plasmid identified by PCR according to Bäumler et al.
† [14]. Outbreak in Thuringia, Germany.

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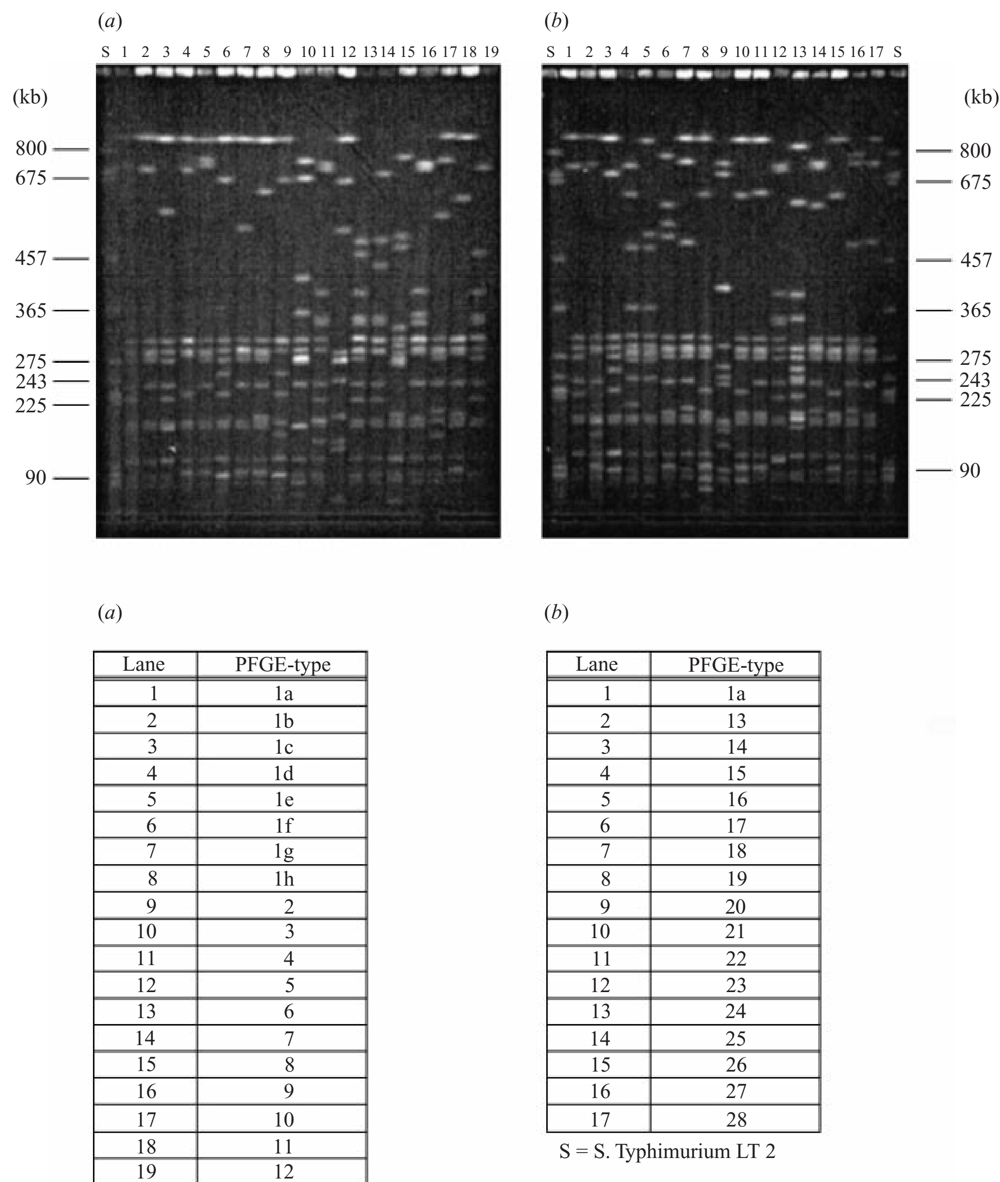

(a)

Lane	PFGE-type
1	1a
2	1b
3	1c
4	1d
5	1e
6	1f
7	1g
8	1h
9	2
10	3
11	4
12	5
13	6
14	7
15	8
16	9
17	10
18	11
19	12

(b)

Lane	PFGE-type
1	1a
2	13
3	14
4	15
5	16
6	17
7	18
8	19
9	20
10	21
11	22
12	23
13	24
14	25
15	26
16	27
17	28

S = S. Typhimurium LT 2

Fig. 2. PFGE patterns identified among *S.* Bovismorbificans isolates.

phonamides. Only a small number of isolates exhibited multiple antibiotic resistance, in particular, strains isolated in Australia.

Ribotypes

Seven different ribotypes could be discriminated among the 162 strains (Fig. 1, see Table 3).

Plasmid profiles

Some of the strains were demonstrated to carry plasmids, either singular small and cryptic plasmids or drug resistant plasmids (as demonstrated by conjugative transfer of antibiotic resistance determinants to an *E. coli* reference strain CV601; data not shown). About 35% of the *S.* Bovismorbificans isolates have been found to carry a 90 kb large plasmid (Table 4) giving rise to a positive pef-PCR reaction. It is interesting to note that this serovar-specific plasmid was mainly detected among the PFGE type 1b strains (Table 4).

PFGE pattern

28 different PFGE patterns (Fig. 2) were identified, however, 85% of the isolates belong to a particular PFGE type designated 1 with some pattern variations (1a, 1b etc.; see Fig. 2a). In Germany this pattern (type 1) was detected among isolates 1993–9 indicating

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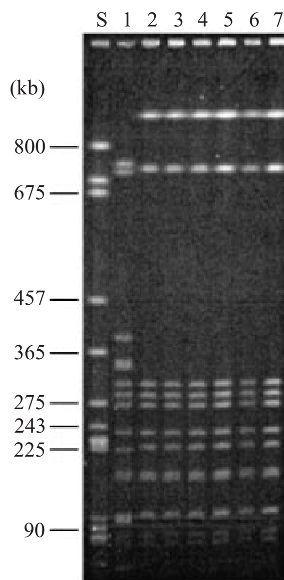

Lane	PFGE-type	Year of isolation
1	23	1999
2	1a	1994
3	1a	1995
4	1a	1996
5	1a	1997
6	1a	1998
7	1a	1999
S	S. Typhimurium LT 2	

Fig. 3. Comparison of PFGE pattern of isolates after several subcultivations and years of storage.

a considerable stability (Fig. 3). Moreover, this pattern type 1 remained stable after several steps of subcultivation.

Variability of the properties

The range of pattern variation within a distinct ribotype and a PFGE type can be seen in Figures 1 and 2 (designated with letters), the variations in plasmid content and antibiotic resistance among defined clonal types are summarized in Table 4. These minor pattern variations noted within a *S.* Bovismorbificans epidemic strain were observed among strains after several steps of subcultivations and therefore regarded as epidemiologically unimportant.

Clonal analysis and designation

Phage types and PFGE patterns were detected as the most polymorphic properties among the *S.* Bovismorbificans strains (Table 5). Both were therefore used to designate clonal types for epidemiological purposes. In combining PFGE pattern and phage types 38 clonal types were identified such as 1-14, 1-32, 7-20, 21-31, etc. (Table 3) demonstrating the great biological diversity among this serovar. However, most of the *S.* Bovismorbificans isolates originating in the various countries (e.g. Australia, Austria, Thailand, Finland, Greece, Sweden, Germany and UAE) and from various epidemiological sources belonged to a limited number of clonal types such as 1-14, 1-20, 1-32. They comprised more than 70% of all isolates.

Table 5. *Comparison of phage types identified among genotypes of S. Bovismorbificans*

Phage type (PT)	PFGE types (see Fig. 2)
PT1	1
PT12	1a, 1b, 1d, 9, 11, 12
PT14	1a, 1b, 1d, 6, 13, 14, 18, 25, 27
PT16	17
PT20	1a, 1c, 7
PT22	1a, 1b, 1c, 1f, 3
PT31	21
PT32	1b, 1e, 1g, 28
UT	1a, 1b, 1h, 4, 7, 17, 19, 22, 23, 24, 26

The genetic distance between all *S.* Bovismorbificans types identified throughout this study has been calculated according to Claus et al. [22] using PFGE-type, ribotype and phage types (Fig. 4).

Epidemiological considerations

Most of the *S.* Bovismorbificans isolates investigated throughout this study belong to clonal type 1-14 associated with single cases in Germany, Austria, Australia, Hungary, and Greece. This clonal type has to be also regarded as the causative agent of a diffuse outbreak in Germany between 1993 and 1997. The second most frequently observed epidemic type, designated 1-32 of *S.* Bovismorbificans was detected among isolates mainly from Finland and Australia, that could be traced back to the consumption of sprouts [7]. The clonal type 1-20 appeared obviously only in Germany and might be a 'local' phage type variation of phage type 14 (see Table 2) whereas the clonal types 1-12 and 1-ut appeared only in Germany and Australia. The clonal types 1-12, 1-14, 1-20, 1-32 and 1-ut include about 78% of all isolates tested. The remaining 12% shared 29 different clonal types such as 3-22, 6-14, 7-20, 9-12, 10-nt, etc. (see Table 3).

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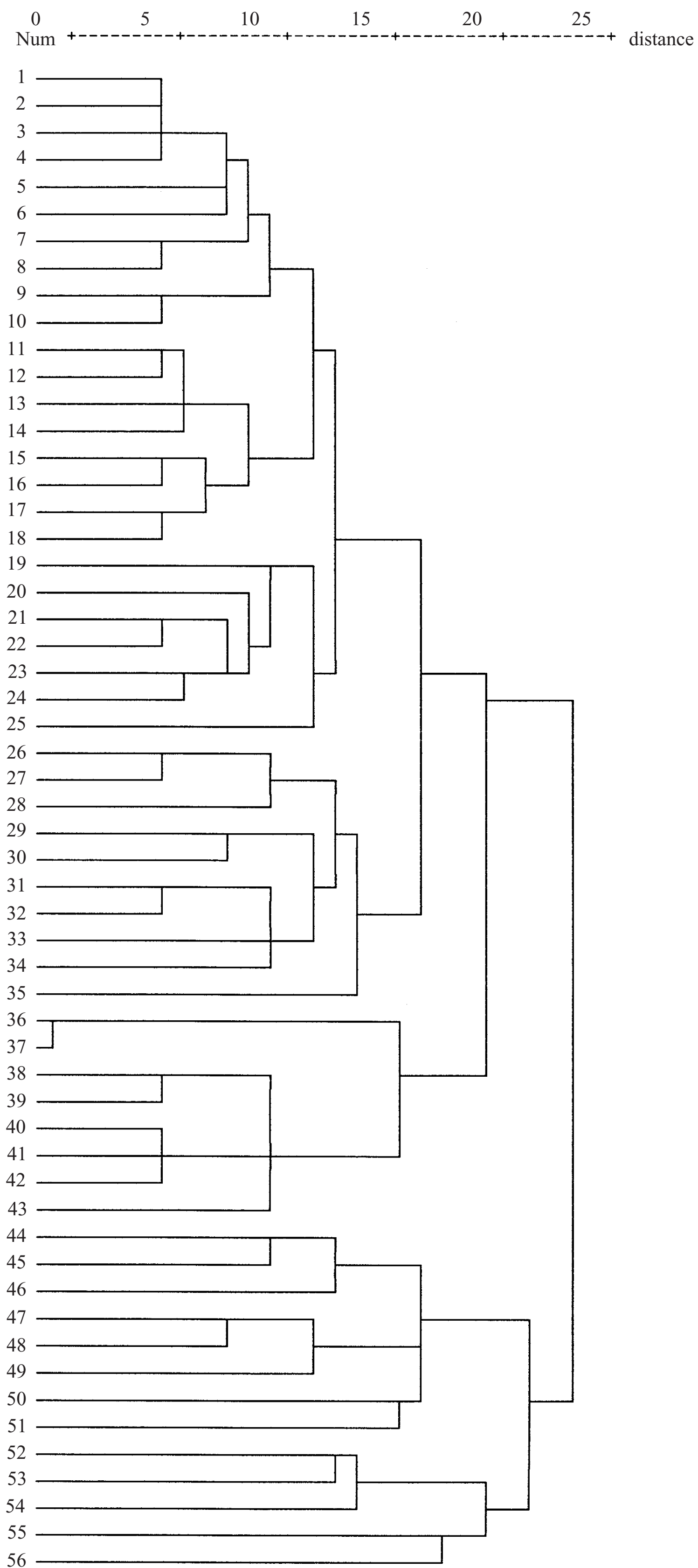

Fig. 4. Genetic distance between the 162 *S.* Bovismorbificans isolates studied throughout this paper according to their PFGE patterns, ribotypes and phage types. (For key see opposite.)

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Remarkable is the clonal relationship between European and Australian strains indicating a common source of infections. However, since overseas travel could be excluded as a common source the increase of *S*. Bovismorbificans in Europe between 1993 and 1998 might be due to the epidemic spread of some international available clonal strains.

DISCUSSION

S. enterica sv. Bovismorbificans (O: 6,8 H:r: 1,5) is a rarely reported salmonella serotype in Europe at least as a causative agent of human gastro-enteritis (but see earlier findings of the sixties [6]. For instance, in Finland, the annual number of *S*. Bovismorbificans, including the cases of domestic and foreign origin, has been around 10, except the outbreak in 1994 where more than 200 cases were diagnosed [3]. In Germany and Netherlands, human cases of *S*. Bovismorbificans were reported frequently in the early 1950s but nearly disappeared in the following years until the early nineties. From 1993/4 to 1997/8 strains of *S*. Bovismorbificans have increasingly been recorded as an emerging human pathogen in European countries [3–5, 19] and became the third most frequently isolated salmonella serovar in Germany [5]. In contrast to Europe, strains of *S*. Bovismorbificans have been isolated rather frequently from animals as well as from humans in Australia. In this country, these strains belong to the top three serovars [8, 9, 20]. Therefore, it seemed appropriate to apply a range of currently available molecular methods (such as PFGE, ribotyping) and phage typing for subtyping *S*. Bovismorbificans in order to trace their epidemic spread, their infection routes and sources as well as their population structure. In particular, phage types and PFGE pattern have been found valuable for the clonal discrimination and therefore for epidemiological grouping of *S*. Bovismorbificans strains (Table 3, Fig. 2).

Significant biological diversity was detected among the 162 *S*. Bovismorbificans strains obtained from Austria, Finland, Sweden, Greece, Hungary, Thailand, Bahrain, UAE, Australia, Great Britain and Germany. Using phage typing together with PFGE typing 36 clonal groups have been established, however, most of the isolates (78%) have been covered by only six clonal types designated 1-12, 1-14, 1-20, 1-22, 1-32, 1-ut (Table 4). Since phage types and PFGE types among the outbreak strains remained stable even after several years under culture conditions (see Fig. 3) and both methods allowed the detection of considerable genetic polymorphism among *S*. Bovismorbificans. Both methods applied in combination, enabled us also to differentiate the 'normal' range of variations (acquisition of plasmid, of antibiotic resistance, slight PFGE pattern variations) from the more genetically distant types (Fig. 4). Interesting to note is the variability of *S*. Bovismorbificans strain with respect to the presence of a 90 kb large plasmid that gave rise to a positive pef-PCR (Table 4, [7]. This type of plasmid has been observed mainly among the Australian strains of PFGE pattern type 1b.

With respect to the source of infection some of the outbreaks could be traced back to sprouts as in Finland [3] and with a larger outbreak in middle Germany (1a-20; see Table 4). In Finland, the two large outbreaks were linked to alfalfa sprouts [4] which were grown from Australian alfalfa seeds. This is confirmed here by establishing the clonal identity of isolates (clonal type 1-32) from Finland and Australia. However, strains of the Swedish outbreak taking place at the same time were found to be of clonal type 9-12, a type that has not been detected among the Australian isolates. Therefore, alternative sources of infections must also be taken in to consideration.

Key to Figure 4.

1	Clonal type 1b-32	15	Clonal type 1a-ut	29	Clonal type 24-ut	43	Clonal type 28-32
2	Clonal type 1b-12	16	Clonal type 1a-22	30	Clonal type 11-12	44	Clonal type 5-RDNC
3	Clonal type 1b-14	17	Clonal type 1a-22	31	Clonal type 17-14	45	Clonal type 15-RDNC
4	Clonal type 1b-ut	18	Clonal type 1a-20	32	Clonal type 18-14	46	Clonal type 10-RDNC
5	Clonal type 1b-22	19	Clonal type 1d-12	33	Clonal type 26-ut	47	Clonal type 16-RDNC
6	Clonal type 1b-22	20	Clonal type 1f-22	34	Clonal type 21-31	48	Clonal type 16-RDNC
7	Clonal type 1e-32	21	Clonal type 1c-22	35	Clonal type 12-12	49	Clonal type 22-ut
8	Clonal type 1g-32	22	Clonal type 1c-22	36	Clonal type 9-12	50	Clonal type 8-RDNC
9	Clonal type 1h-ut	23	Clonal type 1c-22	37	Clonal type 9-12	51	Clonal type 23-ut
10	Clonal type 1h- ut	24	Clonal type 1c-20	38	Clonal type 7-ut	52	Clonal type 14-14
11	Clonal type 1a-14	25	Clonal type 20-RDNC	39	Clonal type 7-20	53	Clonal type 14-12
12	Clonal type 1a-14	26	Clonal type 17-ut	40	Clonal type 27-14	54	Clonal type 3-22
13	Clonal type 1a-14	27	Clonal type 19-ut	41	Clonal type 25-14	55	Clonal type 2-RDNC
14	Clonal type 1a-14	28	Clonal type 4-ut	42	Clonal type 6-14	56	Clonal type 17-16

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It is interesting to speculate that some of the clonal types of the serovar *S*. Bovismorbificans could have been adapted to soil and watery habitats giving rise especially to infections via vegetables [21, 22].

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