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ACADEMIC DISSERTATION

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ABSTRACT

Salmonellosis is one of the most significant zoonoses worldwide and also in Finland. The major serovars causing infections in humans are *Salmonella* Enteritidis and *Salmonella* Typhimurium. *Salmonella* Typhimurium definitive phage type (DT) 1 and *Salmonella* Infantis are considered endemic in Finland. These serovars have frequently caused outbreaks among humans, the source of which is often detected. For the sporadic cases they usually remain unknown. *Salmonella* Agona was not frequently encountered in Finland until a small outbreak among cattle farms occurred in 1994-1995. *S*. Agona became the third most common *Salmonella* serovar in cattle in Finland in 1995. The two more common serovars were *S*. Infantis and *S*. Typhimurium DT1.

Bacterial typing methods are used for outbreak investigations and for surveillance, where the data can be used for risk assessment calculations in addition to the future prevention of outbreaks. In particular the identification of factors that contribute to the persistence and spread of infection in endemic situations, estimations of the effect of animal reservoirs on human cases, and the identification of other risk factors for human infections are among the important reasons for typing.

Salmonella Infantis became more common in cattle in the 1980s, after it spread in the broiler chicken production in Finland in 1971. Subsequently it caused outbreaks among broilers and humans in the 1970s and 1980s. In the 1990s, *S*. Infantis became the predominant serovar among cattle in Finland. In 1995, a feedborne outbreak of *S*. Infantis in cattle occurred. We were able to identify the feedstuff-related genotype by *Xba*I-PFGE methodology. It belonged to the major endemic type, pf1, but differed from it by having a plasmid visible as an intensive band of 60 kb in *Xba*I-PFGE (plasmid subtype pf1/39). Farms infected with the feedstuff-related genotype pf1/39 or the related genotypes pf1/43, pf1/44, pf1/45, or pf1/46 containing the same 60 kb plasmid were also identified. The stability of the feedstuff-related genotype was followed on selected farms. The plasmid was stable on the farms during the follow-up period. The feedstuff-related genotype did not persist in the cattle population. Moreover, there was a general decline in bovine salmonella infections from 1997 onwards.

The genetic diversity of the *S*. Infantis isolates taken from Finnish cattle was also assessed: the *S*. Infantis infection in cattle was highly clonal as 99 per cent of the isolates had *Xba*I-PFGE profiles clonally related to each other. The major genotype pf1 was predominant both at the starting year of our analysis in 1985 and as the infection seemed to fade out in 2003. Traditionally, only one isolate per farm is stored in the national collection. However, an infection may have existed subclinically for a long time in a herd until the first *Salmonella* isolates were obtained. In our analysis of successive isolates from the same herds, we frequently detected minor changes in banding

patterns during long-lasting infections in individual herds. The sampling and testing of several isolates from a herd in outbreak investigations is therefore advisable. There is a trend towards less genetic diversity of the *S*. Infantis infection among domestic isolates from humans and poultry. Up to eight different ribo/IS*200*-types were detected in the 1980s isolates, whereas in the 1990s only two different ribo/IS*200*-types (1A, 1B) were seen. In cattle, 89 per cent of the analysed isolates possessed the ribo/IS*200*-type 1A, although four different ribo/IS*200*-types were recorded in the 1980s isolates, and two ribo/IS*200*-types in the 2000s isolates. The ribo/IS*200*-types and the most common *Xba*I-PFGE profiles determined amongst the analysed cattle isolates could also be detected among domestic isolates from poultry and humans.

After the outbreak in cattle in the years 1994-1995, isolates of *S*. Agona taken from the 1984 to 1999 period were characterized by PFGE using *Xba*I, *Bln*I, *Spe*I, and *Not*I enzymes. Two outbreak-related genotypes, which were not detected in the earlier isolates of *S*. Agona, were identified. Another small possibly genetically related outbreak among cattle farms occurred in 1997. In 1999, a large outbreak of *S*. Agona of domestic origin involving more than 50 human cases occurred. Despite epidemiological investigations carried out by the local authorities, the source of the outbreak remained unknown. Based on our typing data, this outbreak was unrelated to the cattle farm outbreaks, though it did occur in the same region of Finland. The outbreak profile for the 1999 outbreak could not be found in any of the other isolates. As no recent foreign isolates were available, a foreign source of the human infection cannot be disregarded.

Salmonella Typhimurium DT1 has become the most common *S*. Typhimurium phage type among cattle farms in Finland, and has been detected annually since 1980. Domestic and foreign isolates of *S*. Typhimurium DT1 from 1981 to 1999 were characterised. Furthermore, two clusters formed by the effects of the combination of the *Xba*I-, *Bln*I-, and *Spe*I-PFGE profiles, IS*200*-profiles and possession of the serovar-specific virulence plasmid were analysed. The major cluster had no virulence plasmid and included the most common *Xba*I-PFGE profile 10 and IS*200*-profile D, typical of our endemic infection. The results of *Xba*I-, *Bln*I-, and *Spe*I-PFGE gave 54 different combination profiles, which can be applied in the analysis of outbreaks. In contrast, molecular subtyping by *Xba*I-PFGE alone is not discriminatory enough in analysing our endemic infection. The source of sporadic human infections is unknown in most cases, and molecular typing did not reveal any clear infection source. The most common *Xba*I-PFGE profile 10 was also seen in hedgehogs and wild birds. They might act as important reservoirs, maintaining a minimum baseline level of *S*. Typhimurium DT1 in the environment and consequently be possible sources of human infections.

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Nanna Lindqvist

ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AP-PCR	arbitrarily primed polymerase chain reaction
bp	base pair
CHEF	clamped homogeneous electric field
DNA	deoxyribonucleic acid
DT	definitive type
ΔT_m	differences between melting temperature (midpoint in denaturation of DNA)
EDTA	ethylenediamine tetra-acetic acid
EELA	National Veterinary and Food Research Institute
EFSA	European Food Safety Authority
ERIC-PCR	Enterobacterial repetitive intergenic consensus-polymerase chain reaction
ET	electrotype
Evira	Finnish Food Safety Authority
FAFLP	fluorescent amplified fragment length polymorphism
FSCP	Finnish *Salmonella* control programme
F-value	coefficient of similarity-value
G+C	guanine + cytosine
H antigen	flagellar antigen
IS	insertion sequence
kb	kilobase
M	molar (mol/litre)
MAPLT	multiple amplification of phage locus typing
MDa	megadalton
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
MLVA	multiple-locus variable-number tandem-repeats analysis
O antigen	somatic antigen
PCR	polymerase chain reaction
pf	pulsed-field
PFGE	pulsed-field gel electrophoresis
RAPD	random amplified polymorphic DNA
REP-PCR	repetitive extragenic palindromic-polymerase chain reaction
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
SAFLP	single-enzyme amplified fragment length polymorphism
spv	*s*almonella *p*lasmid *v*irulence
UPGMA	unweigthed pair group method using arithmetic averages
UV	ultraviolet light
Vi antigen	surface antigen
VNTR	variable number of tandem repeats

ORIGINAL PUBLICATIONS

The dissertation is based on the following original articles, which are referred to in the text by their Roman numerals (I-IV).

I Lindqvist N., Heinikainen S., Toivonen A-M. and Pelkonen S. (1999)
Discrimination between endemic and feedborne *Salmonella* Infantis infection in cattle by molecular typing.
Epidemiology and Infection, 122: 497-504.

II Lindqvist N and Pelkonen S. (2007)
Genetic surveillance of endemic bovine *Salmonella* Infantis infection.
Acta Vet Scand, 49:15.

III Lindqvist N., Siitonen A. and Pelkonen S. (2002)
Molecular follow-up of *Salmonella enterica* subsp. *enterica* serovar Agona infection in cattle and humans.
Journal of Clinical Microbiology, 40: 3648-3653.

IV Lindqvist N., Heinikainen S., Siitonen A. and Pelkonen S. (2004)
Molecular characterisation of *Salmonella enterica* subsp. *enterica* serovar Typhimurium DT1 isolates.
Epidemiology and Infection, 132: 263-272.

"Epidemiology is defined as the study of the frequency, distribution, and determinants of health and disease in populations (Martin *et al* 1987) and the application of this study to the prevention and control of health problems (Last 1995)".

1. INTRODUCTION

Zoonoses are infections that can spread from vertebrate animals to man. The most important zoonoses in the EU by far are campylobacteriosis and salmonellosis. Salmonellosis is also one of the most significant zoonoses in Finland. It is estimated that about 60 to 80 per cent of the reported cases in humans are of foreign origin. The most common *Salmonella enterica* subsp. *enterica* serovars of domestic origin being *S*. Infantis and *S*. Typhimurium definitive phage type (DT) 1. Both are considered endemic in Finland.

Salmonella Infantis is the predominant serovar in production animals in Finland. Before the introduction of *S*. Infantis into the broiler chicken production in Finland in 1971, only sporadic isolations of *S*. Infantis among animals, animal-feed and humans were made. *S*. Infantis became more common among cattle in the country in the 1980s, and in the 1990s it became the predominant *Salmonella* serovar. In 1995, a feedstuff-related outbreak occurred among cattle farms.

Salmonella Typhimurium DT1 spread to broiler farms in Finland in 1983, and sporadic isolations in broilers have been made since. DT1 has become the most common *S*. Typhimurium phage type among cattle farms, and has been isolated every year since 1980. Annually, DT1 also causes outbreaks among humans.

Salmonella Agona was not frequently encountered in Finland until an increase in the number of isolations found in animals and feed in 1994. A small outbreak of *S*. Agona involving eight cattle farms was seen in 1994-1995, and *S*. Agona was the third most common *Salmonella* serovar in cattle in Finland in 1995, after *S*. Infantis and *S*. Typhimurium. Usually more than 50 per cent of the serovar Agona isolates from humans are of foreign origin, but in 1999 a large outbreak in humans of domestic origin occurred.

In addition to the conventional epidemiological surveys, bacterial typing methods provide the basis for investigations of outbreaks of human and animal salmonellosis today. Typing is also used for surveillance to obtain baseline information and to estimate the effect of animal reservoirs in human cases. Moreover, it is used to identify factors that contribute to the persistence and spread of infection in endemic situations, and to monitor critical points for cross-contamination in food production.

The present study was undertaken to obtain molecular information on the endemic *Salmonella* serovars Infantis and Typhimurium DT1, in addition to the potentially emerging serovar Agona. We

wanted to see the long term genetic changes that can occur during a prolonged endemic infection such as *S*. Infantis, and to evaluate the stability of the molecular profile in individual herds during long-lasting infections. Two outbreak investigations were included in the study to show the usefulness and value of molecular typing in such situations as surveillance. The resulting data can be used for risk assessment for future prevention and investigation of outbreaks. Furthermore, the molecular protocols used can also be adapted for the analyses of other *Salmonella* serovars.

2. LITERATURE REVIEW

2.1 The genus *Salmonella*

The genus *Salmonella* is named after D. E. Salmon, an American bacteriologist and veterinarian, who, together with T. Smith isolated the "hog cholera bacillus" in 1885 (Salmon and Smith 1885). *Salmonella*e belong to the family *Enterobacteriaceae*, and are facultative anaerobic gram-negative straight rods of 0.7 - 1.5 x 2.0 - 5.0 μm in size and shape. They are usually motile, their motility being produced by peritrichate flagella. The colonies are generally 2 - 4 mm in diameter. *Salmonella*e reduce nitrates to nitrites and usually produce gas from glucose and hydrogen sulfide on triple-sugar iron agar. They are indole-negative, urease-negative and usually utilize citrate as a sole carbon source (Brenner 1984). Originally inclusion into the genus *Salmonella* was on the basis that organisms were related to one another antigenically. However, since these organisms have a large number of biochemical characters in common, more emphasis was put on their biochemical activity properties than their respective antigenic structure (Parker 1983). With the development of DNA-based methods, one can now study the genetic relationships in order to decide which organisms belong to the genus.

The nomenclature within the genus was first based on biochemical reactions. Later DNA-relationship studies provided the basis for renaming the subgenera. The genus *Salmonella* was divided by Kauffmann into four subgenera, I - IV (Kauffmann 1966b) on the basis of biochemical reactions. In 1970, Le Minor *et al* re-classified the subgenera as: subgenus I as '*S. kauffmannii*', subgenus II '*S. salamae'*, subgenus III '*S. arizonae'* and subgenus IV '*S. houtenae'*. Later, the four subgenera were shown by DNA-relationship studies to constitute a single DNA hybridization group with five subgroups, where subgenus III was split into DNA subgroups IIIa and IIIb. Le Minor *et al* later identified an additional subgroup (VI). A second DNA hybridization group (Bongor) was identified (Grimont *et al* 2000). In 1982, Le Minor *et al* considered all *Salmonella* serovars to constitute a single species, *S. choleraesuis*, which contained six subspecies (*choleraesuis*, *salamae*, *arizonae*, *diarizonae, houtenae*, *bongori*). Subspecies *indica* was added in 1986. The specific name of the species, *S. choleraesuis*, was also the name of a serovar. Therefore, in 1986 Le Minor and Popoff proposed the new name of *S. enterica*, making the list of the subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori*, and *indica* (Grimont *et al* 2000).

According to the guidelines issued in 1987 by Wayne *et al*, a genomic species is defined as having more than 70 per cent relatedness by DNA-DNA hybridisation with ΔT_m values below 5°C. Using these criteria Reeves *et al* classified two species (*S. enterica* and *S. bongori*) and six subspecies of *S. enterica* (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *indica*) within the genus

Salmonella in 1989. Therefore, the former *S. typhimurium* is now *S. enterica* subsp. *enterica* serovar Typhimurium, or *Salmonella* Typhimurium. Names (usually geographical) are only given to the serovars of *S. enterica* subsp. *enterica* (Grimont *et al* 2000) (www.bacterio.cict.fr/salmonellanom.html).

The identification of *Salmonella* serotypes is based on three kinds of antigen (O, H and Vi) (The Kauffmann-White schema; White 1926, Kauffmann 1941, 1972). The specificity of the somatic (O) antigen, which is heat-stable, is determined by the structure and composition of the lipopolysaccharide of the cell wall. In contrast, the flagellar (H) antigens are heat-labile proteins, flagellins, which are encoded by two genes, *fli*C and *flj*A. These genes determine the antigens of phase 1 and phase 2 flagella: at any given time, either the monophasic (single) or diphasic (two separate) form is expressed (Gillespie and Timoney 1981; Grimont *et al* 2000).

The surface (Vi) antigen is a capsular polysaccharide found in serovars Typhi, Paratyphi C and Dublin (Grimont *et al* 2000). Rough mutants have lost their agglutination capacity to homologous O antiserum.The smooth to rough variation in the O antigen occurs rarely in nature. However, it is common in strains maintained through many generations on ordinary laboratory media. The antigenic formula consists of three parts: the O antigens, the phase 1 H antigen and the phase 2 H antigen (Parker 1983). The different O antigens are designated by Arabic numbers, whereas the H antigens are assigned either with small letters (phase 1 antigens) or Arabic numbers or small letters (phase 2 antigens). In the antigenic formula, the O antigenic factors that are easily modified by mutation are indicated in brackets and those determined by bacteriophages or plasmids are underlined (Grimont *et al* 2000). The antigenic formulae for instance for *S. enterica* subsp. *enterica* serovars Agona, Infantis and Typhimurium are 1,4,[5],12;fgs;[1,2] and 6,7,14;r:1,5 and 1,4,[5],12;i:1,2, respectively.

By 2002, as many as 2541 serovars had been identified, 22 of which are in *S. bongori*. Of the 2519 serovars within *S. enterica*, 1504 serovars corresponded to subspecies *enterica*, 502 to *salamae*, 95 to *arizonae*, 333 to *diarizonae*, 72 to *houtenae* and 13 to *indica* (Popoff *et al* 2004) (www.sciencedirect.com; March 2007).

The G + C content of the *Salmonella* DNA is 50 to 53 per cent (Brenner 1984). The complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2 was published in October 2001 (McClelland *et al*). The size of the sequenced chromosome was 4857 kilobase (kb). The 94 kb virulence plasmid of *S*. Typhimurium strain LT2 was also sequenced. A highly invasive and resistant zoonotic pathogen, *Salmonella enterica* serovar Choleraesuis, was sequenced by Chiu *et al* (2005). For those *Salmonella* strains that have been sequenced so far, the size of the genome

varies between 4460 kb (*S. bongori* 12419 ATCC 43975) and 5091 kb (*S. enterica* Typhimurium SL1344 NCTC 13347) (www.sanger.ac.uk/Projects/Salmonella). The CBS Genome Atlas Database (Hallin and Ussery 2004) is a dynamic database for bioinformatic results and sequence data, including the 4857 kb genome of *Salmonella* Typhimurium LT2.

Plasmids, which are composed of double-stranded circular DNA, are extrachromosomal elements that are often found in the cytoplasm of bacteria. The plasmids may contain antibiotic-resistance genes or virulence-factor genes, such as enterotoxins and adhesins. For instance, the serotype-specific plasmids of *Salmonella*e possess *s*almonella *p*lasmid *v*irulence (*spv*) genes, responsible for virulence in the mouse (Gulig 1990). Plasmids encoding for unknown factors are referred to as cryptic (Tompkins 1992).

2.2 Characteristics of bovine salmonella infections

2.2.1 Clinical features

The disease caused by *Salmonella*e is usually either systemic, or an acute enteritis. In the latter systemic disease is seen only in cases with decreased immune response (Barrow and Wallis 2000). Animals are predisposed to clinical salmonellosis by several stress factors, such as parasitism, viral infections, parturition, poor sanitation, poor nutrition, overcrowding, and transportation. Calves that have not received adequate colostrum within the first 12 hours of life are more susceptible to infection (Gillespie and Timoney 1981). Young animals are also less able to cope with dehydration (Lax *et al* 1995). The pathogenicity of *Salmonella* is both serovar- and host-dependent (Barrow and Wallis 2000). Serovars such as Abortusovis and Pullorum/Gallinarum are adapted to sheep and poultry. Other serovars, such as Dublin and Choleraesuis, cause disease primarily in one animal species (cattle or pigs) but are opportunist pathogens of others (Lax *et al* 1995).

Typical symptoms and signs in the acute phase of the infection are depression, anorexia, fever, weakness and diarrhoea, which may be blood-stained. Pregnant animals may abort. Death occurs most frequently within a week from the onset of clinical signs; in calves often within a day or two. The mortality, usually in the range of 5 to 10 per cent, may be as high as 75 per cent. In calves that survive, there may later be signs of joint infection (Gillespie and Timoney 1981; Wray and Davies 2000). Animals can also be symptomless; they harbour the infection in their lymph nodes or tonsils without excreting the organism in their faeces, but during stress these latent carriers may become active carriers or even clinical cases per se (Wray and Davies 2000). After systemic infection, *Salmonella*e are occasionally excreted in the urine or in a purulent discharge (Parker 1983).

Salmonella Dublin primarily localizes in the placenta and causes abortion, often without any other symptoms or generalized salmonellosis (Lax *et al* 1995). If the calves of these active or latent carriers of *S*. Dublin survive, they may be congenitally infected and may infect contact animals or become carriers themselves. Some cattle in California are reported to have chronic *S*. Dublin infection of the udder, and may shed the organism both in their faeces and milk (Wray and Davies 2000). Calves infected with *S*. Dublin often show respiratory signs, whereas the enteric form predominates for other serovars (Wray and Davies 2000).

2.2.2 Infection routes and epidemiology on the farm

The most important infection route in animals is the faecal-oral (Gillespie and Timoney 1981). Respiratory infection is also possible; *Salmonella* may be spread by aerosols with the use of pressure hoses when cleaning stalls (Lax *et al* 1995; Wray and Davies 2000). Contaminated milk may also cause infections. Occasionally cattle may excrete *Salmonella* in their milk, but more frequently the milk is contaminated by infected faeces during the milking process (Wray and Davies 2000). Newborn calves are exposed to *Salmonella* infection as the carrier cows shed the organism at parturition (Gillespie and Timoney 1981). To cause disease in healthy cattle, oral doses ranging from 10^6 to 10^{11} cells of *S*. Dublin and 10^4 to 10^{11} cells of *S*. Typhimurium are needed (Wray and Davies 2000). However, infection has been seen even after the ingestion of very small doses (less than 10 cells). These infections were associated with ingestion of chocolate in humans and a vegetable fat supplement in cattle (Kapperud *et al* 1990; Jones *et al* 1982).

The transmission of infection from one farm to another is mainly achieved by the purchase of infected cattle (Wray and Davies 2000). Non-symptomatic ruminants shed the bacteria intermittently and therefore infection is difficult to detect (Edrington *et al* 2004). Some asymptomatic carriers continue to eliminate *Salmonella*e in their faeces for weeks, months or years after recovery from a clinical case: and by doing so these carriers contribute to the dissemination of salmonellosis (Brenner 1984). Latent carriers are important in the epidemiology and persistence of *S*. Dublin on farms, because this bacterium can survive in the environment for over a year (Gillespie and Timoney 1981; Wray and Davies 2000). Furthermore, *S*. Typhimurium has been shown to persist in calf rearing units for up to two years, despite cleaning and disinfection routinely carried out between batches (Wray and Davies 2000). Free-living animals, birds, and rodents may also introduce the infection onto the farm (Gillespie and Timoney 1981; Wray and Davies 2000). Cats, dogs, mice, rats, foxes and badgers have been shown to be infected with *S*. Typhimurium. Rats and mice that acquire *S*. Dublin infection do not play a major role in the spread of infection, but they might prolong the persistence of *Salmonella* on farms (Wray and Davies 2000).

Another important infection source for animals is contaminated feedstuffs (Gillespie and Timoney 1981). Both formulated compound feeds and also vegetable proteins, such as soya, have been found to be contaminated with *Salmonella* (Aho *et al* 1996). Imported animal protein, such as fishmeal, and waste from the food industry, might also be contaminated (Wray and Davies 2000). Compound-feed mills may be contaminated by *Salmonella* present in the ingredients, and the presence of *Salmonella* in cooling systems and storage bins may lead to the subsequent contamination of products both during and after processing. To minimize the risk for contamination of stored feed, effective rodent and bird control is important. It has been shown that there is an increased risk of salmonellosis if the storage conditions on the farm are poor. For example, allowing wild birds and cats access to the feed stores (Wray and Davies 2000). In Finland, the Feedingstuff Act has been in force for over 40 years. The purpose of this act is to detect *Salmonella* in feedstuffs. The Finnish Food Safety Authority Evira (the former Plant Production Inspection Centre is also a part) continuously monitors the facilities of feed processors including feed materials, feed mixtures and additives used in feedingstuffs by sampling according to a protocol approved by the Ministry of Agriculture and Forestry. In order to minimize the spread of *Salmonella* from the manufacturing plants to farms, strict control of the raw material for feedstuffs has proved to be most efficient (Ministry of Agriculture and Forestry, Helsinki, Finland, 2000).

In the disease control measures for reducing infection on cattle farms, the monitoring of the environment must be included as *Salmonella*e may survive for prolonged lengths of time in: certain feed, sewage, river and sea water (Murray 2000; Parker 1983). Pastures may be contaminated by polluted water whenever flooding occurs, therefore pastures should not be grazed for four to five weeks after flooding, and surface water should be fenced off. In cases of widespread environmental contamination, such as that in the large western dairy herds in the USA, it is difficult to determine the source of infection (Wray and Davies 2000). Moreover, when investigating disease outbreaks, one must keep in mind that the introduction of infection may sometimes precede the development of clinical disease by several months or years.

2.2.3 Salmonella in cattle in the EU and worldwide

The major *Salmonella* serovars associated with bovine salmonellosis in the EU and elsewhere are serovars Dublin and Typhimurium. The true prevalence of salmonella in cattle in the EU is not known, as there is little systematic screening done. The sampling schemes and diagnostic methods vary between the Member States. The reporting has also been inconsistent for many Member States. Typically the reported data are from clinical salmonellosis outbreaks, so only the most common serovars are reported. For the year 2005, only five Member States of the EU (Estonia, Finland, Italy, Slovenia and Sweden), and also Norway reported data from active monitoring of cattle herds (European Commission 2004; The European Food Safety Authority, EFSA, 2005 and 2006). In 2005, very few, if any, *Salmonella* infected animals or herds were reported in Finland, Norway and Sweden, and less than 1 per cent of the analysed isolates were positive for *Salmonella* in Estonia and Slovenia. In Italy, of the batches of cattle that were investigated prior to slaughter, 6.7 per cent were positive for *Salmonella* (EFSA 2006).

In 2002, the dominating *Salmonella* serovar overall in cattle in the EU was the serovar Dublin (European Commission 2004). In Finland, *S*. Dublin has not been isolated on cattle farms since 1994, when it was isolated on one farm. In the 1960s and 1970s, only sporadic isolations of *S.* Dublin were found. In the 1980s, there was an outbreak of *S.* Dublin among cattle farms in Finland. After that, isolations of *S.* Dublin have only been detected in 1991, on two farms, and in 1994 (National Veterinary and Food Research Institute 1965-2005). However, in Belgium *S.* Dublin is becoming increasingly important (Imberechts and Butaye 2006). *S.* Dublin has been detected both in the USA and Canada. In New Zealand, *S.* Dublin has not been detected in farm animals although it has been isolated in humans. In Australia, *S.* Dublin used to be more frequent in cattle than *S*. Typhimurium. However, since 1990, *S.* Typhimurium is more common in cattle (Wray and Davies 2000).

In 2004, *Salmonella* Typhimurium had become the most common serovar overall in cattle in the EU (EFSA 2005). In 2005, *S.* Typhimurium was found in cattle in 14 of the 25 EU member states; *S.* Typhimurium definitive phage type 1 (DT1) was only detected in cattle in Finland. *S*. Infantis in cattle was found in five member states and *S*. Agona in three states (EFSA 2006). In Norway, *S*. Typhimurium is considered endemic in the country (Heir *et al* 2002).

2.3 Salmonella in Finland

In Finland all *Salmonella* strains isolated from humans are sent by laboratories to the National Public Health Institute in Helsinki, Finland for verification and more accurate epidemiological classification. This practice has been voluntary from the 1960s onwards and compulsory from 1994. Phagetyping of the serovars Enteritidis, Typhimurium and Paratyphi B is also carried out at the National Public Health Institute. This intitute has kept a nationwide register on infectious diseases in humans starting in 1994.

Salmonellosis in humans is relatively rare in Finland: the annual incidence was 44-53 cases per 100 000 inhabitants in 2000-2004, and 85 per cent of these were related to recent foreign travel. The most common serovars causing infection in humans are the serovars Enteritidis and Typhimurium. Most cases of *S.* Enteritidis are of foreign origin whereas the cases of *S*. Typhimurium are mainly (90-95%) of domestic origin. In the 1990s, an average of 350 cases per year of human salmonellosis of domestic origin caused by *S.* Typhimurium was recorded compared with an average 160 cases annually in 2000-2004. *S*. Typhimurium DT1 has been the predominant phage type except in 1997. *S*. Infantis is considered endemic in Finland. Of the isolations made in humans, the majority is usually considered to be of domestic origin. This especially applies to the isolations found in the 1970s and 1980s. The number of *S*. Infantis cases recorded in humans has decreased substantially during the 1990s; an average of 33 cases per year was recorded in 1995-1999, compared with an average only eight cases annually in 2000-2004. An increase in *S.* Agona infections in humans was seen in 1999, with over 80 cases of infection. In the years 2000-2004, an average of 25 cases annually was recorded (National Public Health Institute 1965-1994; Ministry of Agriculture and Forestry 2000; Finnish Food Safety Authority Evira 2006).

Salmonella Agona had been only sporadically isolated from animals in Finland before the small outbreaks on cattle farms in 1994-1995 and in 1997. *S*. Agona was not isolated in any farms in 2001-2005 (Figure 1). *S*. Infantis was rarely [only 4% (20/480) of the *Salmonella* isolations in 1970] encountered among animals before the spread among broiler chickens in 1971. The latter caused an epidemic in both humans and broilers in Finland (Nurmi and Rantala 1972; Rantala 1976). *S*. Infantis became more common among cattle throughout Finland in the 1980s, and in the 1990s it became the predominant *Salmonella* serovar. In recent years, the number of *S*. Infantis infected farms has been five or less (Figure 1). *S*. Typhimurium has been relatively frequently isolated from animals ever since its recording was started in 1965. Phage typing of *S.* Typhimurium isolates according to the Colindale method began in March 1968. Definitive type (DT) 1 was not encountered in isolates obtained from animals before 1980. *S*. Typhimurium DT1 is also

considered endemic in Finland from the 1980s (Figure 1) (National Veterinary and Food Research Institute 1965–2005; Ministry of Agriculture and Forestry 2000; Finnish Food Safety Authority Evira 2006).

Detection of *Salmonella* on a farm or in a food processing plant initiates statutory measures which are always taken. These include epidemiological investigations, disinfection procedures and restrictions on the movement, sale or purchase of animals. The Finnish *Salmonella* control programme (FSCP) was first implemented in May 1995. Its objective is to keep the incidence of salmonella in the production animals in Finland (cattle, pigs and poultry are covered by the programme) and the produce from these held down to the level of no more than 1 per cent. This objective was attained throughout the country in 1995-2004, apart from the year 1999, when 2.2 per cent (64/2939) of the analysed broiler production flocks tested positive for *Salmonella* (Ministry of Agriculture and Forestry 2000; Finnish Food Safety Authority Evira 2006).

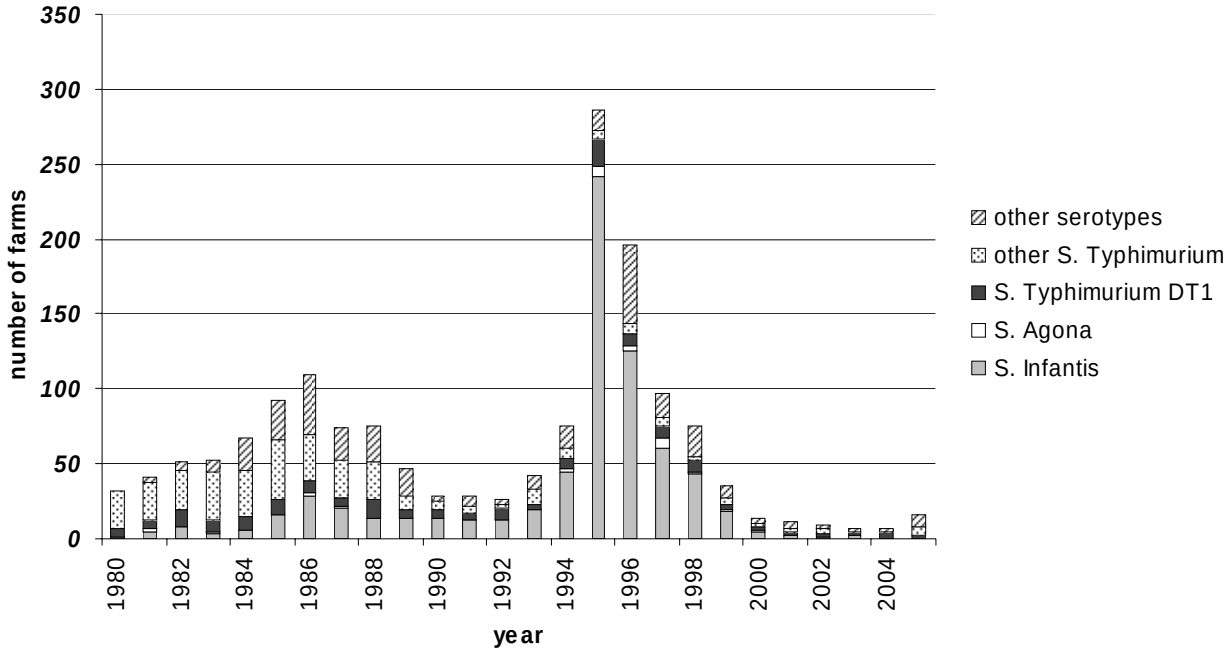

Figure 1. The total number of *Salmonella* infected Finnish cattle farms from 1980 to 2005 showing the number of farms infected with *S*. Agona, *S*. Infantis, *S*. Typhimurium DT1, other phage types of *S.* Typhimurium, and other serotypes.

2.4 Risk assessment and risk management of salmonella infections in cattle

Scientific risk assessments are important tools for estimating the magnitude of different risks and the effects of different interventions to control these risks (Maijala and Ranta 2003). The nature of the risk, the possible consequences, and their probability are evaluated in the risk assessment process (National Veterinary and Food Research Institute 2005). It is based on four stages: hazard identification, hazard characterization, exposure assessment, and risk characterization (Ranta *et al* 2004). The risk assessment begins with *hazard identification*, where the hazard (for example, salmonella) is identified on a general level. The hazard identification can address questions such as what kind of disease the *Salmonella* bacteria might cause, how the disease might spread, and what is the incidence and prevalence of salmonella. The *hazard characterization* includes a more detailed description of the hazard: the microbe itself, the symptoms it may cause, and how it affects its hosts. *Exposure assessment* is calculated based on basic information on the exposure to *Salmonella*. To be able to assess the risk of exposure, transmission models have to be made (Maijala and Ranta 2003; Ranta *et al* 2004). Different exposure models, such as the Bayesian Hierarchial Modeling (Ranta *et al* 2005), are used to assess the exposure or estimate the true salmonella prevalence. A general estimate of the risk of infection is produced by combining information derived from the exposure assessment with information about the dose-response. *Risk characterization* can be done once the risk of infections caused by *Salmonella* has been quantified using the exposure models (Maijala and Ranta 2003; Ranta *et al* 2004).

When risk management actions are evaluated, the true prevalence or incidence should be the basis for evaluation. The Finnish *Salmonella* Control Programme (FSCP) was introduced in 1995, when Finland joined the EU. The aim of the programme was to keep the domestic salmonella prevalence below 1 per cent along the food chain in broiler, turkey, beef, pork, and egg production. The FSCP for cattle is more like a monitoring system than a control programme. The regulations concerning cattle and beef production begin with feed control. All imported, marketed, and manufactured feed materials and compounded feeds are tested for *Salmonella*, and no batches with *Salmonella* detection are approved. The bulls for artificial insemination and their herds of origin are required to test negative for *Salmonella*. Likewise all suspected cattle (with clinical symptoms or epidemiological evidence) are examined bacteriologically. The intention of this sampling and testing of cattle is to limit the spread of *Salmonella* between farms and animals (Tuominen *et al* 2007). If *Salmonella* is detected in cattle, the herd of origin is put under official restrictions, including isolation of *Salmonella*-positive animals and the prohibition of animal movements. Faecal samples from all the animals are examined at one month intervals. The restrictions are lifted when two successive samplings are negative for *Salmonella*.

In the Hazard Analysis and Critical Control Point (HACCP), the hazards or risks and critical control points are determined. Among risk factors associated with salmonellosis in cattle herds are access to feed stores by wild birds, presence of feral cats on the farms, introduction of newly purchased cattle, and lack of isolation facilities (Evans 1996). In a recent study by Fossler *et al* (2005), cattle groups and environmental sample locations likely to be *Salmonella*-positive were assessed on dairy farms in the USA. Cattle-level risk factors associated with *Salmonella* shedding were also evaluated. Larger farms (with at least 100 cows) were more likely to have *Salmonella*-positive cattle compared to smaller farms. Faecal *Salmonella* shedding in cattle was associated with season being more common in summer than in winter. No association was found between *Salmonella* shedding and stage of lactation for lactating cows, parity for any cows, and age (in days) for calves. Therefore few factors at the cattle-level seem to offer means for salmonella control. A potential way of reducing contamination of the farm environment is improved sick pen and maternity pen management, as the sick pen floor, the manure storage area, and the maternity pen were more likely to be *Salmonella*-positive than many other environmental locations.

For control strategies to be successful, the routes and sources of infection and the manifestations of disease must be identified. Control strategies include proper control of rodents and wild birds, improvements in animal feed and production hygiene, sufficient in-house cleaning and disinfection, and salmonella-free replacement animals (Association for Animal Disease Prevention in Finland, 2008).

2.5 Typing methods of *Salmonella* serovars

2.5.1 Phenotypic typing methods

2.5.1.1 Bacteriophage typing

The method is based on the susceptibility or resistance of bacteria to a panel of lytic bacteriophages (i.e. viruses capable of infecting and lysing bacterial cells). The first method developed was the Vi-phage typing method for *Salmonella* Typhi (Craigie and Hjen 1938a, b). In 1943, the *Salmonella* Typhimurium phage typing scheme was established. Using 11 phages, 12 phage types were defined (Felix 1956). In 1959, it was extended to 34 types using 29 phages (Callow 1959).

In 1977, 207 "definitive types" (DTs) of *S*. Typhimurium were defined by using 34 phages (Anderson *et al* 1977). A different typing system, in which typing with another set of bacteriophages is supplemented by biotyping, is used in the Netherlands (Parker 1983, EFSA 2005). Hungary also uses yet a different set of phages (EFSA 2005). Phage typing schemes have also been described for serovars Adelaide, Anatum, Bareilly, Blockley, Braenderup, Bovismorbificans, Enteritidis, Gallinarum, Hadar, Infantis, Montevideo, Newport, Panama, Paratyphi B, Virchow and Weltevreden (Grimont *et al* 2000). Recently a phage typing scheme for *S*. Agona was developed (Rabsch *et al* 2005).

Phage typing is routinely used for subtyping isolates of *S*. Typhimurium as it is one of the basic methods employed in studying the epidemiology of this serovar. Even nowadays, when molecular methods are used for the subtyping of isolates, phage typing is still used. Phage-typing does not require any expensive equipment and is therefore cheap (Grimont *et al* 2000). However, stocks of biologically active phages and control strains need to be maintained. Therefore phage typing is only available at reference laboratories. It is very demanding even for experienced workers, and subject to considerable biological and experimental variability (Maslow *et al* 1993). Moreover, when only a few phage types tend to dominate over a period of time, the discriminatory ability of this typing method becomes diminished. In the 1990s, a multiresistant phage type of *S*. Typhimurium, DT104, emerged and spread in the UK, Denmark, USA and Canada (Kariuki *et al* 1999). In 2004, the dominating *S*. Typhimurium phage types overall among isolates obtained from human infections in the EU were DT104 and DT120 (EFSA 2005).

2.5.1.2 Antimicrobial susceptibility testing

*Salmonella*e used to be susceptible to a wide range of antimicrobials *in vitro*. However, in 1958 resistance to tetracycline was first noted. In 1962, resistance to ampicillin was reported in Britain and rapidly became common. Transferable resistances to streptomycin, tetracycline and sulphonamides were observed in a *S*. Typhimurium strain of phage type 29 from 1958, which by 1968 had acquired a resistance spectrum with resistance to ampicillin, streptomycin, sulphonamide, tetracycline and furazolidone. Resistance to chloramphenicol and kanamycin was also reported (Parker 1983). Plasmids containing genes that confer resistance to antibiotics may readily be acquired by *Salmonella* strains (Brenner 1984). After 1976, resistant strains of *S*. Typhimurium phage types 204 and 193 caused epidemics in calves, with subsequent spread to man. Plasmids coding for resistance to four to six antimicrobials, including chloramphenicol, were carried by these strains (Parker 1983).

Antimicrobial susceptibility testing is relatively inexpensive and easy to use, and the frequent identification of a new or unusual pattern of antibiotic resistance may increase the probability of an impending outbreak. In the identification of the recent worldwide spread of a resistant clone of *S*. Typhimurium phage type DT104 with resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline, antibiotic susceptibility testing played a major role. However, there are multiple genetic mechanisms by which resistance can be acquired from other strains or evolve within a strain, thereby causing phenotypic variation. Different strains may have similar resistance patterns and sequential isolates of the same strain different patterns (Maslow *et al* 1993).

2.5.1.3 Multilocus enzyme electrophoresis (MLEE)

Genes that encode a specific enzyme may be present in one or more forms, and the amino acid composition of the enzyme produced may differ slightly. These differences between isomorphs can be detected electrophoretically (Tompkins 1992). The high degree of polymorphism, which might be seen in soluble cytoplasmic enzymes, may be the result of only one amino acid change. Such polymorphisms can be analysed on large numbers of strains by starch gel electrophoresis. Individual enzymes are stained with the corresponding substrates and indicator dye combinations, the distances of migration of the enzymes are compared, and differently migrating allelic gene products are thus identified. Groups of strains carrying identical allelles are called an electrotype (ET). The genetic distances of different ETs can be calculated based on the proportion of mismatch (Selander *et al* 1986). There tend to be fewer clones and less diverse ETs for the host adapted *Salmonella* serovars than for those serovars that are pathogenic for a variety of host species (Selander *et al* 1990). Genetic diversity and relationships among isolates of eight common serotypes were studied by Beltran *et al* (1988), who suggested that horizontal gene transfer and recombination of chromosomal genes that mediate the expression of cell-surface antigens has been a significant process in the evolution of *Salmonell*ae.

MLEE typing is based on a pattern produced by 20 or more different enzymes. The levels of genetic diversity among clones can also be measured. MLEE is a powerful tool to study the evolutionary relation of pathogenic clones. It has been shown that many bacterial infections are caused by a limited number of pathogenic clones. The major disadvantage of MLEE is that, because it is labour-intensive, it is not practical for routine use (Tompkins 1992).

2.5.1.4 Biotyping

In biotyping, the pattern of activity for 20 or fewer cellular metabolic enzymes is established. Such data can be routinely obtained if automated systems for species identification are available in the laboratory. Variation in gene expression is most commonly the reason for isolates of the same strain to differ in one or more biochemical reactions. However, random mutations may also alter the result (Maslow *et al* 1993). Strains of the same serovar might show different sugar fermentation patterns, which are determined by the presence or absence of enzymes, and hence genetically determined. For example, the xylose+ and xylose- character of *S*. Typhi may be of epidemiological interest (Brenner 1984). The utilization of d-tartrate in serovar Paratyphi B is used to separate biotype Java that is associated with diarrhoea, from biotype Paratyphi B that is associated with paratyphoid. Biotype Java can utilize d-tartrate whereas biotype Paratyphi B cannot (Grimont *et al* 2000).

In many serovars, a subdivision according to the biochemical character yields only a small number of identifiable biotypes. This is because a significant number of strains behave differently in only a few biochemical tests (Parker 1983). Although biotyping is considered highly reliable, the discriminatory power of biotyping is generally low. If multiple isolates of an unusual serovar are detected an outbreak can be identified effectively, and occasionally epidemic strains manifest unique biotypes (Maslow *et al* 1993). In conventional biotyping, the results are expressed after a definite incubation time. When the rate of each reaction is read, and the kinetics of each evaluated, a biochemical fingerprint of an isolate is obtained (Möllby *et al* 1993).

2.5.2 Molecular genetic typing methods

2.5.2.1 Plasmid profile

The first genotypic method used for strain separation within *Enterobacteriaceae* was plasmid profiling (Riley and Cohen 1982). Bacteria of the same clonal line are expected to carry the same plasmids since copies of the resident plasmid are distributed between the two daughter cells when the bacterium divides (Olsen 2000). For strains that lack plasmids or have only one or two of them, plasmid profiling has poor discriminatory power. Other potential problems arise from the fact that plasmids are extra-chromosomal elements. Plasmids carrying resistance determinants may spread rapidly from one strain to another and persist for prolonged periods (Maslow *et al* 1993). However, in strains with plasmids coding for drug resistance, plasmids can be lost if antibiotic selective pressure is withdrawn. Consequently, interpretation of typing results may be difficult (Threlfall *et al* 1994).

The acquisition or loss of a plasmid serves as a marker for recent events (Tompkins 1992). In a study by Brown *et al* (1992), isolates of *Salmonella enterica* over six successive generations from a broiler breeding farm were analysed by plasmid profiling. In each successive generation, an increase in the diversity of plasmid profiles was seen. However, all the profiles observed could be derived from one of the existing profiles by the acquisition or loss of a single plasmid. The conclusion of the study was that persistence and cross-infection rather than reintroduction was the cause of the persisting serovar Berta infection.

The stability of plasmids during storage has been assessed by Olsen *et al* (1994a). They found plasmids to be stable if the *Salmonella* strains were stored at -80°C and the higher the temperature the larger the risk of loss of plasmids. The total number of plasmids lost increased with storage time when stored at room temperature (22°C to 30°C). Casalino *et al* (1984) noted that plasmids of *S*. Wien remained stable over a period of more than ten years. In a natural outbreak, caused by *S*. Berta, a succession of different plasmid profile types was detected (Olsen *et al* 1996a)

Plasmids in a size range of 2 to 150 kb are often carried by strains of *Salmonella*, but variations between serovars occur regarding the size distributions and frequencies (Olsen 2000). Plasmid analysis is only of limited use in *Salmonella* serotypes in which the majority of the isolates contain only one plasmid, e.g. serovars Dublin and Enteritidis. However, it has been particularly useful for discrimination within certain phage types, such as 49 and DT204c, of *S*. Typhimurium (Threlfall *et al* 1994). In a study by Olsen *et al* (1990), 99 per cent of the analysed strains of *S*. Dublin carried plasmids, but only nine different plasmids profiles were recorded thus limiting the usefulness of plasmid profiling in epidemiological studies. Recently, an outbreak investigation on multiresistant *S*. Typhimurium DT104 showed that plasmid profiling was more discriminatory than the high-resolution genotyping methods of PFGE and fluorescent AFLP (Lawson *et al* 2004). In evaluating molecular typing methods for the analysis of *S*. Typhimurium DT104 isolates from healthy pigs, plasmid profiling was found to be superior to PFGE, RAPD and IS*200*-typing (Malorny *et al* 2001). Plasmid profiles have also been used successfully for strain discrimination in epidemiological investigations of outbreaks caused by *Salmonella* serovars such as *S*. Gold-coast (Threlfall *et al* 1986a), *S*. Berta (Sørensen *et al* 1991; Olsen *et al* 1992), and *S*. Livingstone (Chrichton *et al* 1996). By the development of other genotyping methods, plasmid profiling has lost some of its importance.

2.5.2.2 Plasmid fingerprinting and identification of plasmid-mediated virulence genes

Discrimination between plasmids of similar molecular mass, or determination of the degree of molecular relatedness between plasmids of different size, may be achieved by digesting the plasmids with a restriction endonuclease and comparing the number and size of the restriction fragments generated (Maslow *et al* 1993; Threlfall *et al* 1994). The same restriction pattern should be seen in identical plasmids (Grimont *et al* 2000), but the results may be extremely difficult to interpret if a strain carries multiple plasmids (Threlfall *et al* 1994). However, a study by Rankin *et al* (1995) demonstrated the importance of plasmid fingerprinting, as plasmid analysis, on its own, can easily lead to the assumption that plasmids of the same molecular weight are the same plasmids. Their work showed plasmids of the same molecular weight could be unrelated whereas plasmids of different molecular weight could be closely related. In a study by Browning *et al* (1995), considerable plasmid diversity and evolutionary divergence was detected in the serotype-specific plasmid of *Salmonella* Dublin. Baggesen *et al* (1992) observed that plasmid profiling followed by restriction enzyme analysis was useful as it showed variations in the serotype-specific plasmid and also the presence of co-migrating plasmids of the same size as the serotype-specific plasmid.

Plasmids carried by certain serotypes (serotype-specific plasmids) possess a highly conserved common area which carries genes responsible for the virulence of the serotype to certain strains of mice (Williamson *et al* 1988). A DNA probe specific for these *s*almonella *p*lasmid *v*irulence (*spv*) genes makes rapid screening of a large number of strains possible. In epidemiological investigations, the presence or absence of serotype-specific plasmids can be useful (Threlfall *et al* 1994). At least 12 serotypes contain virulence plasmids. The size of the plasmids range from 54 to 98 kb, and phage type differences were found in plasmid carriage within a serotype (Lax *et al* 1995).

2.5.2.3 Ribotyping

Restriction fragment length polymorphism (RFLP) associated with the ribosomal operon and detected by Southern blot analysis is called ribotyping (Stull *et al* 1988). Initially ribotyping, detecting variations in the copy number and location of 16S and 23S rRNA (*rrn*) loci, was used as a taxonomic tool (Grimont and Grimont 1986). The complete nucleotide sequence of a 16S ribosomal RNA gene obtained from *E. coli* was determined by Brosius *et al* and published in 1978. Stull *et al* (1988) tested the ability of *E. coli* rRNA to hybridize with heterologous DNA purified from genetically diverse gram-negative bacteria. These authors found cross-hybridization to occur between the *E. coli* rRNA probe and unrelated species. Therefore a probe derived from the *E. coli* ribosomal operon can be used widely (Maslow *et al* 1993).

In the 1990s, ribotyping was widely used for the typing of many *Salmonella* serovars. In general, restriction polymorphisms in the areas surrounding the seven rRNA operons in *Salmonella* are highlighted by ribotyping (Olsen 2000). In a study by Altwegg *et al* (1989), ribotyping provided additional information and further differentiation of selected *S*. Typhi strains that were of the same phage type but not related otherwise. However, the sensitivity was dependent on the restriction enzymes used to digest the chromosomal DNA. Ribotyping also proved valuable in another analysis of *S*. Typhi, where a total of 31 different ribotypes were detected among isolates from both sporadic cases and a large, well-defined outbreak (Navarro *et al* 1996). Analysis of strains of *S*. Dublin by IS*200*-typing, ribotyping, PFGE, RFLP and plasmid profiling demonstrate that ribotyping was the most discriminatory method (Olsen and Skov 1994). Ribotyping was used to trace the source and extent of spread of human infections caused by *S*. Livingstone (Crichton *et al* 1996). Guerra *et al* (1997) evaluated five different restriction endonucleases for optimising the ribotyping of *S*. Typhimurium for epidemiological and phylogenetic purposes. In their system, ribotyping had higher typability and sensitivity compared to phage typing. For both sporadic and epidemic isolates of *S*. Brandenburg, only two ribotypes were seen (Baquar *et al* 1994a). Five different ribotypes were found in isolates of *S*. Infantis, although ribotyping alone was not efficient in differentiating between various infection sources (Pelkonen *et al* 1994). Ribotyping of ten representative strains of *S*. Typhimurium DT193 yielded three different ribotypes. However, the 16S *rrn* profile could not distinguish between *S*. Typhimurium and *S*. Stanleyville (Baquar *et al* 1994b). Ribotyping of selected strains of 33 phage types of *S*. Enteritidis gave eight ribotypes, as strains of several phage types had the same ribotype (Olsen *et al* 1994b). In a study of 32 strains of *Salmonella* serogroup D1 (O antigen group 9) eleven distinct 16S rRNA gene profiles were observed, seven of which were specific to individual serotypes (Stanley *et al* 1994). However, Ezquerra *et al* (1993) concluded that although the 16S rRNA gene profiles may differ within a serovar, they are also sometimes common to many serovars.

The fingerprints provided by ribotyping can be both easy to interpret and to reproduce (Threlfall *et al* 1994). Nevertheless, the discriminatory power of ribotyping is limited, because the ribotypes are generally a relatively stable characteristic within a species and epidemiologically unrelated isolates sometimes have the same pattern (Maslow *et al* 1993). On the other hand, when ribotyping is applied in combination with other DNA-based typing methods such as IS*200* fingerprinting and PFGE, its discriminatory power is increased and it can be suitable for discriminating between strains both within serotype and phage type (Threlfall *et al* 1994).

2.5.2.4 IS*200*-typing

Insertion sequences (IS) are mobile genetic elements that contain only the genes that are necessary for their own transposition. In 1983, Lam and Roth (1983) described a 708 base pair *Salmonella*-specific insertion sequence, IS*200*. This insertion sequence has been shown to be distributed on conserved loci on the chromosome of many *Salmonella* serotypes in copy numbers ranging between one and 25 (Gibert *et al* 1990). IS*200*-typing is based on the number and distribution of IS*200* elements in the genome. It has been found to be more valuable for differentiation within serotypes where phage typing schemes do not exist, such as for *S*. Brandenburg and *S*. Infantis (Threlfall *et al* 1994). In combination with ribotyping, it is an especially suitable method of discrimination for epidemiological purposes (Threlfall *et al* 1994) unless the copy number happens to be low and only a few bands are generated (Stanley and Saunders 1996).

The absence of IS*200* has been noted in strains of *S*. Agona, *S*. Daressalam and *S*. Hadar (Olsen 2000). However, Fantasia *et al* (1997) found IS*200*-typing to be an extremely useful tool for discriminating clones of *S*. Hadar in their study on isolates from sporadic and epidemic cases. Differentiation within the phage type could not be achieved for *S*. Enteritidis. On the other hand, discrimination has been achieved both within serotype and phage type for *S*. Typhimurium (Threlfall *et al* 1994). Distinct genotypes for *S*. Paratyphi B and *S*. Java were distinguished by IS*200*-typing, with 13 unique IS*200*-profiles, for the majority of strains shared the predominant 16S rRNA profile (Ezquerra *et al* 1993). In a study on 32 selected isolates of *Salmonella* serogroup D1 (O antigen group 9), Stanley *et al* (1994) observed 20 different IS*200*-profiles though four isolates had no copies of the insertion sequence at all. In *S*. Infantis, for which either phage typing or plasmid profiling per se had not been applicable, the combination of ribotyping and IS*200*-typing generated 15 genotypes. Separately, the two methods gave five and 11 profiles, respectively (Pelkonen *et al* 1994). In *S*. Dublin, only one IS*200* pattern was seen in the 35 strains examined (Olsen and Skov 1994). The pattern obtained was identical to that of a previous study on bovine and human isolates of *S*. Dublin from England and Wales. In *S*. Abortusovis, IS*200*-typing proved more reliable than plasmid profiling. It also provided information on the geographic origin of the strains, since the IS*200*-profiles were less polymorphic in isolates from the same area (Schiaffino *et al* 1996).

IS*200*-typing was more valuable than ribotyping in differentiating isolates of *S*. Montevideo (Old *et al* 2000). In *S*. Glostrup isolates, both IS*200*-typing and ribotyping proved valuable, as seven and three different profiles were detected, respectively (Old *et al* 1999). Neither IS*200*-typing nor ribotyping proved very valuable for a majority of the analysed isolates.

IS*200*-typing in *S*. Thompson yielded five different profiles whereas ribotyping yielded 10 profiles. However, 84 per cent of the isolates were of the same ribotype. This major clone was IS*200*-negative, but in analysing the other isolates IS*200*-typing was found to be highly discriminatory (Chisholm *et al* 1999). In *S*. Typhi, IS*200*-typing is not very valuable for strain discrimination. Although in a study by Navarro *et al* (1996), eight different IS*200*-profiles were obtained, one profile being predominant. Similarly, IS*200*–typing has not been valuable for the analysis of *S*. Gallinarum either. The majority of the analysed strains had the same IS*200*-profile irrespective of their biotype (Olsen *et al* 1996b). In a study on a novel serotype of *Salmonella* (4,12:a:-), ribotyping was more discriminatory than IS*200*-typing (Chrichton *et al* 2000).

The fingerprints provided by IS*200*-typing can be both easy to interpret and reproduce, but the analysis might be indiscriminate particularly within phage type. In addition IS*200*-elements are not possessed by all serotypes or phage types (Threlfall *et al* 1994). Even so, in taxonomic studies of relationships between and within *Salmonella* serovars, IS*200*-typing has often been used (Olsen 2000).

2.5.2.5 Pulsed-field gel electrophoresis (PFGE)

PFGE was first described by DeMarini and Fuscoe in 1991. PFGE is a variation of agarose gel electrophoresis where two electric field orientations are alternated. Linear fragments of DNA ranging from less than 10 kb to more than 6 Mb can be separated with excellent resolution (Threlfall *et al* 1994). Clamped homogeneous electric field (CHEF) electrophoresis is the most complex configuration of this technique. In CHEF, an array of hexagonally arranged electrodes is used to generate uniform electric fields at an angle of 120° to each other. This ensures the straight line migration of large DNA fragments through the gel (Grimont *et al* 2000). For PFGE, restriction enzymes that cleave the chromosomal DNA into between 5 and 20 fragments ranging in size from 10 to 800 kb are used. Typically, distinct well-resolved fragments representing the entire bacterial chromosome are shown in one gel (Maslow *et al* 1993). PFGE has been chosen as the method used in both PulseNet US, an American collaboration for surveillance of food-borne pathogens (http:/www.cdc.gov/pulsenet), and the European Enternet, where the typing data from different sources and different countries are compared (Gatto 2006). The PFGE protocol is standardized for all laboratories participating in the network, and PFGE profiles are stored in a central database. Although highly discriminative for *Salmonella*, PFGE is time-consuming and require effort in the analysis and comparison of restriction profiles (Grimont *et al* 2000).

Epidemiological analyses of *Salmonella*e are often done using combinations of several typing methods, such as phage typing (when applicable), ribotyping, IS*200*-typing, plasmid profile and

PFGE. PFGE has been used both for subdivision within serotype such as *S*. Brandenburg (Baquar *et al* 1994a), *S*. Berta (Ellis *et al* 1998), and *S*. Javiana (Lee *et al* 1998) and phage type such as *S*. Typhi (Nair *et al* 1994), and *S*. Enteritidis (Lukinmaa *et al* 1999).

S. Agona does not carry IS*200* (Gibert *et al* 1995) and is unlikely to be subdivided by ribotyping (Threlfall and Hampton, unpublished). However, PFGE proved useful in the analysis of an international outbreak caused by *S*. Agona contaminated snacks (Threlfall *et al* 1996). In investigating an outbreak of human salmonellosis caused by *S.* Infantis, PFGE typing results indicated a common source for the outbreak (Wegener and Baggesen 1996). In characterizing human and environmental isolates of *S.* Infantis, 35 distinct PFGE profiles were detected. None of the environmental isolates shared the profile common to all the human isolates, therefore the source for the human outbreak could not be identified (Murakami *et al* 1999). PFGE results indicated a spread of a single strain of *S*. Infantis among humans in Argentina (Merino *et al* 2003). Moreover, PFGE proved to be very useful for subdivision of strains of *S*. Typhimurium definitive type (DT) 12, 193 and 104 in an outbreak. In particular, the PFGE profiles of DT12 and DT193 were identical, whereas DT104 yielded a clearly different profile (Corbett-Feeney and Ni Riain 1998). In a study by Kariuki *et al* (1999), isolates of 11 different phage types of *S.* Typhimurium were divided into eight PFGE clusters. No consistent pattern of association between the phage types of the isolates and a particular PFGE cluster was seen.

A problem reported infrequently in some serovars such as *S*. Saintpaul, is the presence of unspecific nuclease activity (Baggesen *et al* 1996). This phenomenon makes the preparation and analysis of PFGE profiles impossible. Recently, Liesegang and Tschäpe (2002) determined that the DNA is degraded during electrophoresis by Tris radicals in the running buffer. By adding thiourea to the Tris-containing buffer, the degradation of the DNA could be prevented.

In a study of 110 *S. enterica* subspecies *enterica* isolates and 25 serotypes genotyped by multilocus sequence typing (MLST), PFGE and amplified fragment length polymorphism (AFLP), PFGE was found to be as discriminatory as AFLP. Moreover, PFGE fingerprints were easier to interpret and reproduce, in addition to being less time-consuming to analyse. Similarly, it is easier to compare PFGE profiles between laboratories, and therefore PGFE is the preferred molecular typing method for surveillance and outbreak investigations (Torpdahl *et al* 2005). In typing 85 clinical isolates of *S*. Typhimurium, PFGE was superior to MLST, as MLST could reveal no differences between the nucleotides within the four selected genes in the analysed isolates (Fakhr *et al* 2005). However, for the typing of the highly homogenous *S*. Typhimurium DT104 in outbreak investigations, even PFGE is not discriminative enough (Murphy *et al* 2001).

2.4.2.6 Polymerase chain reaction (PCR) - based typing methods

Random amplified polymorphic DNA (RAPD) typing, arbitrarily primed PCR (AP-PCR)

RAPD or AP-PCR is based on the amplification of DNA fragments, in which the primer is not directed at a known genetic locus. The single short primer is typically 10 base pairs in length, and result in the amplification of one or more unpredictable loci. The number and size of the PCR generated set of fragments is the basis for the typing of the isolates (Maslow *et al* 1993). Although the typing method is fast and simple, the reproducibility has been variable (Threlfall *et al* 1994). It is affected by the thermal cycler and DNA polymerase variations (Meunier and Grimont 1993). However, by using a commercially available PCR buffer optimization kit, standardization of thermal cycling parameters and selection of discriminatory primers, a highly discriminatory and reproducible characterization of *Salmonella* isolates can be achieved (Hilton *et al* 1997). Despite this, the between-laboratory reproducibility remains a problem in RAPD (Grimont *et al* 2000).

The first published studies on the use of RAPD or AP-PCR in the analysis on *Salmonella*e were on isolates of *S*. Enteritidis. In two studies, better discrimination between the isolates were obtained by RAPD than by phage typing (Fadl *et al* 1995) or phage typing, ribotyping and PFGE (Lin *et al* 1996). Another study showed that RAPD was useful in discriminating isolates of *S*. Enteritidis in outbreak investigations both on its own, and as a supplement to phage typing and PFGE (Skibsted *et al* 1998). One primer used in the RAPD analysis of 89 *Salmonella* isolates belonging to 22 serotypes, produced fingerprints that discriminated between different isolates but did not discriminate between serotypes. Another primer produced a pattern shared by 35 isolates from 12 serotypes. It is also difficult to match fingerprints from different gels by computer (Burr *et al* 1998). For these reasons, Lim *et al* (2005) concluded that a combination of RAPD and ERIC-PCR could be more useful in differentiating strains of *Salmonella* spp than a combination of two different RAPDs. In a comparative study three genotyping methods (automated ribotyping, PFGE and RAPD) were used to analyse 32 isolates of *S*. Livingstone. The discriminatory ability in addition to the reproducibility for RAPD was found to be low. However, RAPD was both rapid and inexpensive, compared to PFGE, and might therefore be suitable for screening purposes (Eriksson *et al* 2005).

Enterobacterial repetitive intergenic consensus (ERIC)-PCR

A highly conserved, repetitive element, about 126 bp in length, was identified in *Salmonella*. However, the chromosomal locations of these enterobacterial repetitive intergenic consensus (ERIC) sequences may differ between and within different species and strains, providing a basis for typing (Grimont *et al* 2000). The DNA fingerprints obtained by ERIC-PCR are not very complex, and making finer distinctions between the strains are more difficult (Versalovic *et al* 1991).

The use of ERIC-PCR for subtyping *Salmonella*e was first described by Kerouanton *et al* (1996), but it could not subdivide 32 strains of *S*. Dublin. In a study by Burr *et al* (1998), ERIC-PCR produced unique fingerprints for almost all 89 *Salmonella* isolates belonging to 22 serotypes, but these fingerprints did not identify the serotypes. Furthermore, ERIC-PCR was found to be valuable in the analysis of *S*. Typhimurium, *S*. Virchow, *S*. Enteritidis, *S*. Abortusequi, *S*. Choleraesuis, *S*. Bareilly and *S*. Dublin (Bennasar *et al* 2000; Chmielewski *et al* 2002; Saxena *et al* 2002). However, it was not able to differentiate between Argentinian strains of *S*. Infantis (Merino *et al* 2003).

Repetitive extragenic palindromic (REP)-PCR

Repetitive extragenic palindromic (REP) consensus sequences of 38 bp have been identified in *Salmonella*, and among other enteric bacteria. Fingerprints of different bacterial genomes can be produced by using these sequences as efficient primer binding sites in PCR reactions. The complex DNA fingerprints are reproducible and diagnostic for specific strains (Versalovic *et al* 1991).

REP-PCR was first used for the typing of *Salmonella*e in 1996, when only one type was detected in 32 strains of *S*. Dublin (Kerouanton *et al* 1996). In contrast, REP-PCR was useful in the analysis of isolates of *S*. Saintpaul, *S.* Typhimurium, *S.* Virchow, *S.* Enteritidis and *S*. Infantis (Beyer *et al* 1998; Bennasar *et al* 2000; Chmielewski *et al* 2002; Merino *et al* 2003). Modified REP-PCR typing with the ERIC2 and BOXA1R primers was highly reproducible in the retrospective analysis of an outbreak of *S*. Infantis. It also distinguished between the serovars (Johnson *et al* 2001). In a study with 68 *Salmonella* isolates of ten different serovars, both PFGE and REP-PCR were able to differentiate among isolates of the same serovar, but REP-PCR using BOX, ERIC, and REP primers had a greater discriminatory capacity than PFGE in differentiating closely related isolates. It was suggested that REP-PCR would therefore be the preferred method in transmission studies of *Salmonella*, in those cases in which an individual isolate needs to be traced back to a specific source (Weigel *et al* 2004). Woo and Lee (2006) also suggested that REP-PCR (using ERIC and REP primers) may be preferred to PFGE as the most suitable method. In another study, REP-PCR with the primers ERIC and $(GTG)_5$ were compared with each other with regard to their discriminatory power between serotypes. The reproducibility was poor between different PCR runs

and one serotype did not always correlate to only one ERIC or $(GTG)_5$ fingerprint. However, the fingerprint heterogeneity within a serotype was limited. As REP-PCR produced fingerprints of nontypeable strains, it can reveal additional information when serotyping is not possible (Rasschaert *et al* 2005).

PCR-restriction fragment length polymorphism (RFLP)

In PCR-RFLP, a specific fragment is subjected to PCR amplification and the amplified DNA is subsequently digested with restriction enzymes. This restriction profile is more reproducible than the pattern obtained by RAPD (Olsen 2000).

A study demonstrated that using PCR-RFLP primers annealing to regions of the bacterial rRNA operon yielded unique electrophoretic patterns of the *Hinf*I digested PCR products in the six serotypes of *Salmonella enterica* analysed (Shah and Romick 1997). Hong *et al* (2003) concluded that PCR-RFLP of 32 flagellin genes (24 phase 1 and eight phase 2 genes) using restriction endonucleases *Sau*3A and *Hha*I was fast and accurate in identifying the serotype of 112 *Salmonella* isolates.

Amplified fragment length polymorphism (AFLP)

The AFLP method is based on selective PCR amplification of restriction fragments of genomic DNA without prior sequence knowledge (Vos *et al* 1995). The restriction fragments are generated by two restriction enzymes, with 4-bp and 6-bp recognition sites. Then the template DNA for PCR amplification is generated by ligating double-stranded adapters to the ends of the DNA restriction fragments. The restriction fragments are selectively amplified using primers which contain adapter-defined sequences. The amplified fragments are detected by denaturing polyacrylamide gel. The pattern obtained is highly reproducible, and the patterns can be scored by automatic reading of gels and direct transfer into software programs (Olsen 2000).

In the first published study on the use of AFLP in *Salmonella*e, 78 different *Salmonella* strains and 62 serotypes were analysed. All serotypes had unique profiles, and AFLP also enabled phage type identification (Aarts *et al* 1998). AFLP analysis on 89 strains of *Salmonella* including both species *S. bongori* and *S. enterica* in addition to all subspecies of *S. enterica* showed that AFLP is useful in studies on population structure in *Salmonella* (Torpdahl and Ahrens 2004). In addition, AFLP was found to be more discriminatory than ribotyping and PFGE in the analysis of *S.* Typhi strains (Nair *et al* 2000). In another study, AFLP differentiated between *S*. Typhimurium phage types DT9 and DT135, and the resulting polymorphic bands could be used for subtyping within both phage types (Lan *et al* 2003). Molecular markers using AFLP were tested on 121 isolates of 33 phage types of *S*. Typhimurium. These markers correlated with phage type distribution thereby showing a potential

to replace phage typing in the future (Lan *et al* 2007). In a longitudinal study of 18 pig farms over a three-year period, AFLP differentiated between distinct clones within DT104. It was also more discriminatory than the PFGE and REP-PCR methods (Gebreyes *et al* 2006). In a study on *S*. Typhimurium comparing three PCR-based methods, AFLP, PCR-phage typing and detection of integrons, the highest discriminatory power was achieved with the use of AFLP (Mikasová *et al* 2005).

A single-enzyme approach (SAFLP) based on the use of only one restriction enzyme with a single adapter was used to fingerprint 30 strains of *S. enterica* belonging to 14 different serotypes. SAFLP was able to differentiate between the serotypes and also to differentiate between both the phage types and between individual strains. Another advantage of SAFLP, besides its specifity, is its reproducibility and speed. It is faster to perform than many other DNA-based methods (Peters and Threlfall 2001). Fluorescent AFLP (FAFLP) uses fluorescent dye-labeled PCR primers. It may provide important insights into the microepidemiology of different *Salmonella* serovars (Lawson *et al* 2004). FAFLP was applied to 46 isolates of *S*. Typhimurium, comprising nine phage types. This method is highly discriminatory and was capable of grouping most serovar Typhimurium isolates according to phage type (Hu *et al* 2002). In a study by Tamada *et al* (2001), *S*. Typhimurium strains were analysed by both fluorescent AFLP and PFGE; both methods were equally useful for epidemiological typing, though no data on the phage type was given. In a study on 97 strains of *S. enterica* subsp. *enterica*, FAFLP had a discriminatory capacity equal to that of PFGE (Lindstedt *et al* 2000). In another study, FAFLP showed a greater ability than PFGE to discriminate between outbreak-associated and epidemiologically unrelated isolates of *S*. Typhimurium DT126. However, neither method was sufficiently sensitive to separate all epidemiologically unrelated DT126 isolates from the outbreak isolates (Ross and Heuzenroeder 2005a). When investigating an outbreak of multiresistant *S*. Typhimurium DT104, the genotype of the outbreak-associated strain could not be differentiated from that found in most multiresistant DT104 isolates by either PFGE or fluorescent AFLP (Lawson *et al* 2004). When analysing 110 isolates and 25 serotypes of *S. enterica* subspecies *enterica* with multilocus sequence typing (MLST), PFGE and AFLP, it was concluded that with the MLST scheme used, PFGE and AFLP had a higher discriminatory power. It was suggested that AFLP should be used for local outbreak investigations, as the interpretation of the AFLP fingerprints was very subjective and dependent on the person performing both the analysis and the interpretation (Torpdahl *et al* 2005).

IS*200*-PCR

IS*200*-PCR is a procedure for the amplification of DNA fragments with outward-facing primers complementary to each end of the insertion sequence IS*200*. The method was evaluated and compared with other molecular methods such as ribotyping, RAPD analysis, ERIC-PCR and PCR

ribotyping (Millemann *et al* 2000). The results for IS*200*-PCR were in accordance with those of the other typing methods for the analysed isolates of *S*. Typhimurium, but no data on the phage type was given.

Multilocus sequence typing (MLST)

In MLST, a set of housekeeping, ribosomal, and/or virulence-associated genes are amplified by PCR. Usually housekeeping genes are used. Then automatic sequencers are used to determine the nucleotide sequences of approximately 400-bp regions of at least seven genes. The data provided by MLST is similar to that obtained by multilocus enzyme electrophoresis (MLEE) except in much greater detail, as MLST has the ability to assess individual nucleotide changes (Kotetishvili *et al* 2002; Ross and Heuzenroeder 2005b). MLST is considered a useful tool for studying evolution and global epidemiology of *Salmonella*e (Ross and Heuzenroeder 2005b), and in surveillance at both the national and international level, as the technique is very reproducible and can easily be exchanged between laboratories. However, the technique is less useful for local epidemiological surveillance and outbreak investigations, as it is not discriminatory enough (Torpdahl *et al* 2005).

MLST using four genes (16S rRNA, *manB*, *pduF*, *gluA*) was able to differentiate between strains of several PFGE types in a study on 231 *Salmonella* isolates grouped into 22 serotypes and 12 strains of undetermined serotype. Moreover, MLST typed some isolates that were untypable by PFGE. MLST with these particular genes was more discriminatory than PFGE as it detected all genetic variation within the amplified gene fragments. In contrast, PFGE examined only those changes that occurred in the cleavage sites for the particular restriction enzyme used. On the other hand, PFGE randomly 'probes' the entire genome (Kotetishvili *et al* 2002). When 110 isolates of 25 serovars of *S. enterica* subspecies *enterica* were investigated by the MLST method using seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA, thrA*), that lacked diversity, the ability to discriminate between isolates was lower than that obtained with the other methods used (PFGE and AFLP) (Torpdahl *et al* 2005). In a comparison study a total of 85 clinical isolates of *S*. Typhimurium from cattle were analysed by PFGE and MLST. The selected genes used were three housekeeping (*manB*, *pduF*, *glnA*) and one virulence (*spaM*) gene. No nucleotide differences between the tested isolates could be detected by MLST (Fakhr *et al* 2005). On the other hand, Sukhnanand *et al* (2005) analysed 25 *S. enterica* isolates, of five different serotypes mainly from cattle. The MLST targeted five housekeeping (*panB*, *icdA*, *manB*, *mdh*, *aceK*) and two virulence (*fimA*, *spaN*) genes. This study found MLST was discriminative both between and within serovar, including *S*. Typhimurium. One must therefore conclude that the choice of the targeted genes is crucial in obtaining discrimination between isolates.

Genomic sequences derived from temperate phages in S. Typhimurium were used to design primer sets, which were used to analyse sequence variations of prophage loci. The typing was based on the presence or absence of an amplified product. This multiple amplification of phage locus typing (MAPLT) was more discriminatory than PFGE and MLST with the housekeeping genes (*fhuA*, *sucA*, *tonB*, *manB, glnA*), regardless of phage type (Ross and Heuzenroeder 2005b).

Multiple-locus variable-number tandem-repeats analysis (MLVA) and variable number of tandem repeats (VNTR)

The variable number of tandem repeats (VNTR) belong to a class of repetitive DNA that appears to contain a high level of polymorphism, thereby giving the VNTR-based typing a high discriminatory capacity (Lindstedt *et al* 2003). The multiple-locus variable-number tandem-repeats analysis (MLVA) is based on capillary separation of multiplexed PCR products from VNTR loci in the bacterial genome. These PCR products are labeled with fluorescent dyes (Lindstedt *et al* 2003, 2004). The VNTR-based method has a greatly superior discrimination within the highly homogenous *S*. Typhimurium DT104 phage type compared to those of *Xba*I PFGE, AFLP and integron pattern analyses (Lindstedt *et al* 2003). These authors also observed a high correlation between MLVA clusters and PFGE clusters of *S*. Typhimurium (Lindstedt *et al* 2003, 2004). However, the MLVA method is not suitable for predicting phage types, as it does not group all the similar phage types within the same MLVA clusters, even though a correlation with the *S*. Typhimurium phage types was found (Lindstedt *et al* 2004). MLVA was effective in identifying a regional outbreak of *S*. Typhimurium DT12 in Denmark and locating its source, whereas the routinely used PFGE did not discriminate between the isolates (Torpdahl *et al* 2006). MLVA was evaluated, and compared with PFGE and phage typing, for subtyping *S*. Enteritidis. It was concluded that MLVA was more discriminatory than PFGE and phage typing among non-epidemiologically in addition to epidemiologically linked isolates. MLVA also was found to have good reproducibility (Boxrud *et al* 2007).

2.5.2.7 DNA microarray

The DNA microarray methodology allows assessment of differences and changes in bacterial genomic contents. It has been widely used for comparative *Salmonella* research since genomic comparison of *S. enterica* serovars and *S. bongori* were performed by Chan *et al* in 2003. Porwollik *et al* (2004) found that gene contents sometimes differed more within a serovar than between serovars. The *Salmonella* strains that share a distinct profile of gene content are called genovars. Two distinct groups among 13 strains of serovar Typhimurium DT104 were identified by a prototype DNA microarray developed for strain differentiation (Pelludat *et al* 2005). Cooke *et al* (2007) used microarrays to detect genes that exhibit significant genetic variation in *S*.Typhimurium,

and that could be used for discrimination between field isolates. Prophage sequences were found to be hot spots of genome variation, and multiplex PCR assays were designed to distinguish between *S*. Typhimurium isolates cost-effectively. Although epidemiological application of DNA microarray is still in its infancy, the technology will no doubt lead to improved molecular genetic typing methods in the near future.

2.5.3 Trends in typing of *Salmonella*e

To evaluate any possible trends in genotyping of *Salmonella*e and to see the development and overall popularity of certain phenotypic and molecular typing methods in research on *Salmonella*, searches on Medline on a selected typing method and salmonella were conducted in March 2007 (http://www.ncbi.nlm.nih.gov and www.pubmed.gov). DNA microarrays are not included as a typing method although they are currently widely used. They are mainly used in non-epidemiological typing studies. The use of various typing methods including the first publication from 1950 onwards is shown in Table 1. The use of both phenotypic and molecular typing methods over time is shown in Figures 2A and 2B. Apart from the last time period, which is from 1.1.2005 to March 2007, each time period is of five years duration starting at 1950-1954 for the phenotypic methods. For genotypic methods start at 1980-1984 onwards. Many new genotypic methods were developed in the early 1990s, with ribotyping, IS*200*-typing and PFGE. However, the introduction of PCR strongly increased the number of various, PCR-based, typing methods available. Judging by the number of new publications after the introduction of a new typing method, the overall popularity of PFGE as a first choice of typing method seems fairly clear. PFGE has also been chosen as the 'Gold Standard' typing method in internationally standardized protocols, such as PulseNet and Enternet. Some typing methods never seem to have gained any further popularity (e.g. the phenotypic methods MLEE and biotyping, or several PCR-based molecular typing methods). However, for the very recent and promising PCR-based typing methods, such as MLST, MLVA and VNTR, it is still too early to say. The same applies to the DNA microarray methodology.

Table 1. The overall use of various phenotypic and molecular typing methods in typing *Salmonella* serovars from 1950 onwards. Results of a Medline search on the methods and salmonella conducted on 13th March 2007. The total number of hits and the first publication for *Salmonella* typing are shown.

Typing method	**Number of hits**	**First publication**
Bacteriophage typing	893	1950; Scholtens RT[a]
Biotyping	67	1956; Levi E
Antimicrobial susceptibility testing	110	1977; Balzer K
Multilocus enzyme electrophoresis	32	1988; Beltran P *et al*
Plasmid profiles	225	1982; Riley, Cohen
Plasmid fingerprinting	100	1982; Taylor *et al*
IS*200*-typing	61	1991; Stanley *et al*
Ribotyping	146	1993; Esteban *et al*
PFGE	607	1994; Olsen *et al*
AP-PCR	7	1995; Fadl *et al*
RAPD-PCR	51	1996; Lin *et al*
ERIC-PCR	13	1996; Kerouanton *et al*
REP-PCR	22	1996; Kerouanton *et al*
Identification of *spv* genes	4	1997; El-Gedaily *et al*
PCR-RFLP	22	1997; Shah, Romick
AFLP	12	1998; Aarts *et al*
IS*200*-PCR	1	2000; Millemann *et al*
MLST	10	2002; Kotetishvili *et al*
VNTR	7	2003; Lindstedt *et al*
MLVA	5	2004; Lindstedt *et al*

a The phage typing scheme for *Salmonella* Typhimurium was established in 1943.

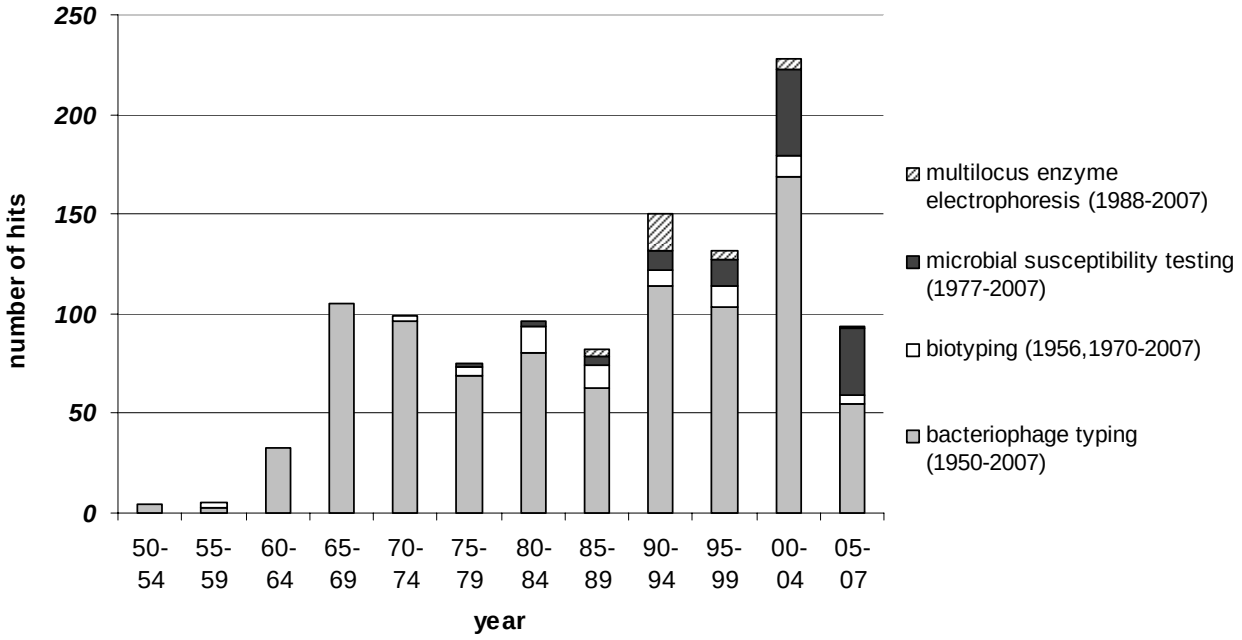

Figure 2A. The use of phenotypic typing methods in typing *Salmonella*e over time. Results of Medline search on the methods and salmonella was conducted in March 2007. The total number of hits within the time period and the years when the method has been in use according to the Medline records are shown.

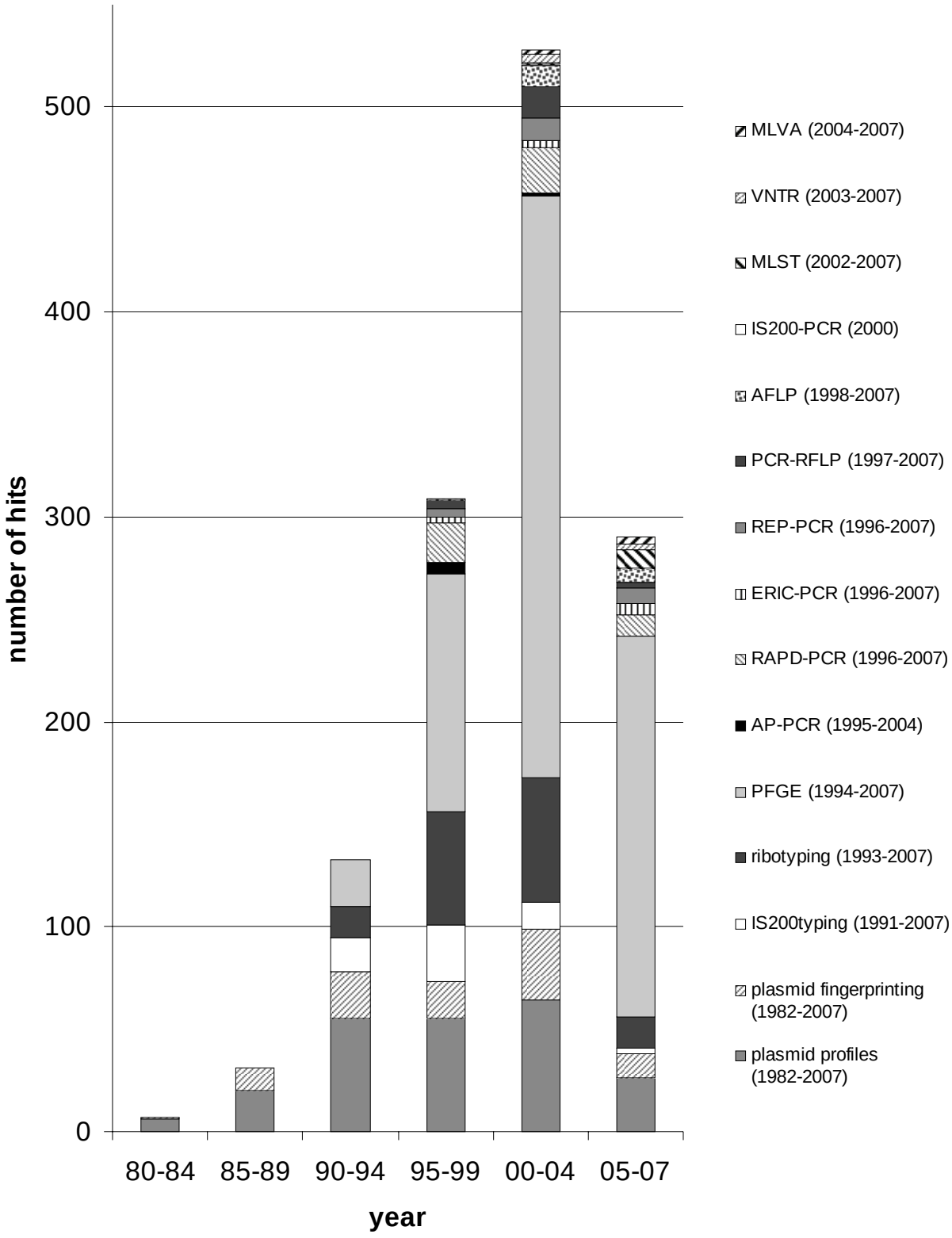

Figure 2B. The use of molecular typing methods in typing *Salmonella*e over time. Results of Medline search on the methods and salmonella was conducted in March 2007. The total number of hits within the time period and also the years when the method has been in use according to the Medline records are shown.

3. AIMS OF THE STUDY

The aim of this work was to understand the molecular epidemiology of salmonella infections in cattle caused by the major endemic serovars *Salmonella* Infantis, *S*. Agona and *S*. Typhimurium DT1, and thereby to:

- identify risk factors for human infections,
- identify infection sources for outbreaks,
- follow the persistence of particular infections,
- recognize new infections, and
- assess the efficacy of control measures.

The specific aims of the study were to:

1. Identify the genotype of the feedstuff-related *S*. Infantis outbreak strain and detect the farms that might have got their *S*. Infantis infection from the contaminated feed in 1995, and to follow the stability of the feedstuff-related genotypes on selected farms (I).

2. Describe the genetic diversity among *S*. Infantis isolates obtained from Finnish cattle over two decades, follow the persistence of the feedstuff-related outbreak strain from 1995 in the cattle population, and learn about the genetic variation of *S*. Infantis isolates in individual herds with long-lasting infections (II).

3. Analyse, whether the *S*. Agona infection in cattle was genetically related to the infection in humans by molecular means, particularly the outbreak among humans in 1999 (III).

4. Characterize the *S*. Typhimurium DT1 infection in cattle, other animals and in humans to identify sources of human infections (IV).

4. MATERIAL AND METHODS

4.1 MATERIAL

The *Salmonella* isolates were mainly obtained from the collections belonging to the Finnish Food Safety Authority Evira (the former National Veterinary and Food Research Institute, EELA, is a part of Evira), Helsinki, Finland, and to the National Public Health Institute, Laboratory of Enteric Pathogens, Helsinki, Finland. Phage typing of *S*. Typhimurium isolates was carried out at the National Public Health Institute in Finland, where it began by the Colindale method in March 1968. *Escherichia coli* strains V517 (35.6, 4.8, 3.7, 3.4, 1.8, 1.4 MDa) (Macrina *et al* 1978) and 39R861 (98.0, 42.0, 23.9, 4.6 MDa) (Threlfall *et al* 1986b) were used as plasmid reference strains.

4.1.1 *S*. Infantis isolates (I, II)

The isolates (total no = 659) were obtained from the Finnish Food Safety Authority Evira in Helsinki, Finland. This includes the former National Veterinary and Food Research Institute (EELA) in Helsinki, and its regional laboratories in Oulu, Seinäjoki and Kuopio (n=655; 636 isolates from cattle from 478 farms, and 19 isolates from feed). The Plant Production Inspection Centre in Vantaa (n=4) is also now part of Evira.

In the former province of Vaasa (where most *S.* Infantis isolations from cattle were made in the 1990s) as early as 1971 the first isolations were found in: imported soya feed, hygiene samples in local slaughterhouses, pig, cattle, and a broiler rearing facility. In the subsequent years, isolations were obtained from poultry slaughterhouses and in 1976 from imported slaughterhouse waste. From 1978 onwards, cattle isolations have been obtained every year (Aho *et al* 1996; National Veterinary and Food Research Institute 1965-2005) (II: table 1).

The analysed *S*. Infantis isolates from cattle originated from farms (n=588; 382 faecal and 206 other isolates), slaughterhouses (n=15) and slaughter transportation vehicles (n=33) from 1985 to 2003. Apart from carcass-samples from the slaughterhouse, the farm isolates were mostly found in faecal samples. The samples from slaughterhouses and cattle transportation vehicles were hygiene samples and therefore not to be traced to any specific farm. The cattle feed isolates originated from the feed production plant (n = 5) and cattle farms (n = 18). The cattle farm isolates were representative of the geographical distribution of *S*. Infantis positive farms (II: figure 2). Two or more isolates taken from 142 farms during the eradication of the infection were available for analysis. If available, isolates from faecal samples were analysed. The isolates were either obtained over the same year or from several years. All isolates had been serologically confirmed to be *S*. Infantis and stored at -70°C, except for the isolates obtained during the 1980s, which were stored on egg agar slopes prior to analysis.

4.1.2 *S*. Agona isolates (III)

The isolates (total n = 110) were obtained from the Finnish Food Safety Authority Evira in Helsinki, Finland [the former National Veterinary and Food Research Institute (EELA) in Helsinki, and its regional laboratories in Oulu, Seinäjoki and Kuopio (n=62) and the Plant Production Inspection Centre in Vantaa (n=2) are now part of Evira], from the National Public Health Institute, Helsinki, Finland (n = 41), from the Laboratory of Fur Animal Association, Vaasa, Finland (n = 2) and from two commercial feed producing companies in Finland (n = 3).

Salmonella Agona was not isolated in Finnish production animals until 1975, when it was detected in one sample obtained from a turkey. In 1976, it was isolated in a horse and also a hen. The next encounter was in 1981, when two cattle farms were found to be *Salmonella* positive. In 1983, *S*. Agona was detected on one cattle farm. In 1981, 1982, and 1984, *S*. Agona was isolated in samples obtained from foxes. In 1985, it was isolated in a dog. In 1986 and 1987, it was isolated in two cattle farms. In 1988, it was isolated in a dog and also a hen. Then no isolations were detected until 1994, when a cat tested positive and, at the end of the year, two cattle farms tested positive. The first outbreak among cattle in Finland occurred in 1994-1995 and involved eight farms. Another small outbreak among cattle occurred in 1997. Of the cattle farms tested after the first outbreak, four, seven, two, one and one were positive for *S*. Agona for the years 1996, 1997, 1998, 1999 and 2000 respectively. After 2000, no farms tested positive for *S*. Agona (National Veterinary and Food Research Institute, 1985-2005).

The analysed *S*. Agona isolates were obtained from cattle, animal feed, fur animals, humans and other sources during the years 1984 to 1999. Isolates collected from the outbreak among cattle in 1994-1995 in addition to other cattle isolates (n = 32) from 1984–1999 were analysed. In addition, isolates (n = 28) from imported products, feedstuff, fur animals and others, and sewage water were also analysed. The isolates from humans provided by the National Public Health Institute were classified either as domestic (a patient had not been abroad during the month preceding the infection) or foreign. Thirty of the human isolates had been associated with recent foreign travel. Isolates from sporadic cases of domestic origin from 1996 and 1997 and three isolates from an outbreak with over 50 human cases in 1999 were also analysed. All isolates had been serologically confirmed to be *S*. Agona and stored at -70°C, except for the isolates obtained in the 1980s, which had been stored on egg agar slopes prior to analysis.

4.1.3 *S*. Typhimurium DT1 isolates (IV)

The isolates (total n = 255) were obtained from the National Public Health Institute, Helsinki, Finland (n = 140) and from the Finnish Food Safety Authority Evira (EELA is a part of it), Helsinki, Finland (n = 115). The isolates were obtained from humans, animals, feed, or the environment from 1972 to 1999. They were chosen among all available isolates of *S*. Typhimurium DT1 based on details of origin (animal species, date or year of isolation, geographical location) in order to get as representative a material as possible. In practice the isolates had to be widely distributed both in time and location. The isolates from humans from 1972 to 1999 were classified either as domestic (a patient had not been abroad during the month preceding the time when the specimen was taken) or foreign based on the recent travelling history of the patient. However, in judging whether the origin of an isolate is domestic or foreign in origin, there is always the risk of misclassification. Most of the isolates of foreign origin were from countries to which tourism from Finland is common. Travellers might have already had the *Salmonella* bacteria when leaving home for the respective destination. The *Salmonella* isolation might have been carried out after returning to Finland, and the isolate threfore classified as 'foreign' based on the basis of recent foreign travel. Such a misclassification might be easier to detect as S. Typhimurium DT1 is rare as an infection source in many countries. The domestic isolates were isolates obtained from outbreaks (for the period 1972 to 1988). The other isolates were obtained from outbreaks or sporadic cases (1990 to 1999). In either case, all isolates had been serologically confirmed to be *S.* Typhimurium, phage typed as DT1 and stored at -70°C, except for the isolates obtained in the 1980s, which had been stored on egg agar slopes prior to analysis.

4.2 METHODS

4.2.1 Pulsed-field gel electrophoresis (PFGE) (I-IV)

Preparation of PFGE-samples (I-IV). Chromosomal DNA was prepared in gel blocks as described previously by Birren and Lai (1993), but with some modifications. Bacteria were harvested from 1.1 ml of an overnight culture. Agarose (1% low melting) was used for the plugs (InCert Agarose, FMC BioProducts, Rockland, ME, USA). Lysozyme treatment at 37°C for 4 hrs was followed by proteinase K at 50 °C for 20 hrs. The plugs were stored in 0.5 M EDTA at 4 °C.

*Xba*I and S1-nuclease digestion for PFGE (I-IV). The agarose plugs were dialysed against 10 mM Tris, pH 7.4, 1 mM EDTA, 50 mM NaCl before treatment. Restriction enzyme and S1-nuclease analyses were performed using slices from the same plugs. The slices were digested at 37°C for 16 - 18 hrs, with 20 units of *Xba*I in the reaction buffer supplied by the manufacturer (New England

Biolabs, Beverly, MA, USA). The reaction was stopped with 0.5 M EDTA, pH 8.0. To linearise the plasmids (Barton *et al* 1995) other slices from dialysed plugs were treated with 2 units of S1-nuclease in the reaction buffer supplied with the enzyme at 37°C for 45 min (Promega, Madison, WI, USA; code M576/1,2), after which EDTA was added.

*Sfi*I and *Spe*I digestion for PFGE (*S*. Infantis) (I,II). In *Xba*I-PFGE, there was only a one-band-difference between the two most common PFGE profiles. Therefore additional analyses were performed for isolates of these two profiles. The slices were digested with 20 units of *Sfi*I at 50°C for 16 - 18 hrs or 12 units of *Spe*I at 37°C for 16 - 18 hrs in the reaction buffer supplied by the manufacturer (New England Biolabs, Beverly, Mass., USA).

*Not*I, *Spe*I and *Bln*I digestion for PFGE (*S*. Agona) (III). The slices were digested with either 20 units of *Not*I or 12 units of *Spe*I in the reaction buffer supplied by the manufacturer (New England Biolabs, Beverly, MA, USA) at 37°C for 16 - 18 hrs. Digestion with 5 units of *Bln*I was carried out in the reaction buffer supplied by the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany) at 37°C for 4 hrs.

*Spe*I and *Bln*I digestion for PFGE (*S*. Typhimurium DT1) (IV). The slices were digested with 10 units of *Spe*I in the reaction buffer supplied by the manufacturer (New England Biolabs, Beverly, Mass., USA) at 37°C for 16 - 18 hrs. Digestion with 10 units of *Bln*I was carried out in the reaction buffer supplied by the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany) at 37°C for 4 hrs.

Performing of PFGE (I-IV). PFGE was performed using clamped homogeneous electric field (CHEF) electrophoresis (CHEF-DRIII, Bio-Rad, Melville, NY). The agarose slices were loaded into 1 per cent agarose gels (FastLane, FMC Bioproducts, Rockland, BM, USA) and subjected to electrophoresis in 0.5 x Tris-borate-EDTA buffer (Sambrook *et al* 1989) at 14°C for 19 hrs, pulse ramp time 2 - 30 sec (*Xba*I and S1-nuclease) (*S*. Infantis), voltage 6 V/cm, reorientation angle 120°. The pulse ramp time was 10 - 30 sec for *S*. Agona and *S*. Typhimurium (*Xba*I and S1-nuclease). The pulse ramp time was 1 - 20 sec for the *Not*I and *Spe*I digested plugs and 10 - 40 sec for the *Bln*I digested plugs (*S*. Agona). The pulse ramp time was 5 - 15 sec for the *Spe*I digested plugs and 10 - 40 sec for the *Bln*I digested plugs (*S*. Typhimurium). The pulse ramp times used for the few isolates of S. Infantis digested with *Sfi*I were 0.5 - 5 sec for 16 hrs and 1 - 13 sec for 17.5 hrs, whereas the time intervals for *Spe*I were 2 - 30 sec for 19 hrs and 1 - 13 sec for 17.5 hrs.

All the gels were stained with 0.5 μg/ml ethidium bromide and photographed by a Polaroid MP-reprocamera. Bacteriophage lambda concatamers (New England Biolabs, Beverly, MA, USA) were used as molecular weight standards. The molecular weights of the linearised plasmids and restriction fragments were determined by plotting the distance of migration against the $\log_{10}$ of the molecular size fragments (Sambrook *et al* 1989; Tenover *et al* 1995). DNA profiles differing by one or more DNA fragments larger than 125 kb were assigned a pulsed-field (pf) type number. When discrimination of the *Xba*I-PFGE analysed isolates was based on all visible DNA fragments, regardless of their size and intensity, the pf-types could be further divided into plasmid subtypes (*S*. Infantis) (I, II). DNA profiles differing by one or more DNA fragments were assigned a pulsed-field (pf) type number (*Xba*I restriction) or letter (other enzymes) (*S*. Agona) (III). DNA profiles differing by at least one fragment larger than 20 kb were assigned a PFGE profile number (*S*. Typhimurium) (IV). The coefficient of similarity values (F) between the pf-types were calculated as described (El-Adhami *et al* 1991). After visual analysis of the PFGE profiles, a computer program for analysis of electrophoretic patterns (GelCompar, Applied Maths, Kortrijk, Belgium) was used to generate dendrograms (Vauterin and Vauterin 1992).

4.2.2 Plasmid analyses (I-IV)

Plasmids smaller than 20 kb were isolated by the alkaline lysis method as described (Grinsted and Bennett 1988). The preparations were analysed in 0.9 per cent agarose gels (SeaKem LE, FMC Bioproducts, Rockland, ME, USA), 4 V/cm, for 1.5 h in 1 x Tris-acetate-EDTA buffer (Sambrook *et al* 1989) and the gels were stained with 0.5 μg/ml ethidium bromide. Plasmids larger than 20 kb were analysed by pulsed-field gel electrophoresis (PFGE). The PFGE agarose plugs were treated with S1-nuclease, which linearises plasmids (Barton *et al* 1995) thereby making plasmid analysis and size determination easier. S1-nuclease treatment was carried out in the reaction buffer supplied with the enzyme (Promega, Madison, WI, USA; code M576/1,2) at 37°C for 45 min. The analyses were performed using slices from the same agarose plugs used for chromosomal profiling by PFGE (see above). *Escherichia coli* strains V517 (35.6, 4.8, 3.7, 3.4, 1.8, 1.4 MDa) (Macrina *et al* 1978) and 39R861 (98.0, 42.0, 23.9, 4.6 MDa) (Threlfall *et al* 1986b) were used as plasmid reference strains in all alkaline lysis isolation procedures.

For restriction fingerprinting (*S*. Infantis) (I) plasmid preparations were treated with 10 units of *Hind*III, *Sfi*I or *Xba*I restriction enzyme (New England Biolabs, Beverly, MA, USA) and analysed in 1 per cent PFGE agarose gels, with the pulse ramp times of 0.1 - 2.0 sec, 9 V/cm, at 14°C, 120° reorientation angle, in 0.5 x Tris-borate-EDTA buffer for three hrs.

4.2.3 Ribotyping and IS*200*-typing (*S*. Infantis, *S*. Typhimurium DT1) (I, II, IV)

Ribotyping and IS*200*-typing were performed as described by Pelkonen *et al* (1994). Bacterial DNA was isolated as described previously (Stull *et al* 1988) and digested with *Ban*I; *Eco*RI digestion (New England Biolabs, Beverly, MA, USA) was used to confirm some IS*200*-types (Pelkonen *et al* 1994) (*S*. Infantis) (I, II). Bacterial DNA was digested with *Bam*HI, *Ban*I, *Pst*I, *Pvu*II, or *Sma*I (New England Biolabs, Beverly, MA, USA). *Pvu*II, which lacks restriction sites within the 16S *rrn* gene, provided the optimal resolution of bands and was used for ribotyping. *Pst*I, which lacks restriction sites within IS*200*, provided the clearest resolution of IS*200* bands and was used for the IS*200* analysis (*S*. Typhimurium) (IV). For ribotyping, a 1.3 kb PCR product of the *Escherichia coli* 16S *rrn*B operon was amplified and purified as described previously (Pelkonen *et al* 1994). For IS*200*-typing, a 557-bp PCR product of the IS*200* insertion sequence was amplified from our own *Salmonella* serovar Infantis isolate K1469 with the primer pair 5'-CCTAACAGGCGCATACGATC-3' and 5'-ACATCTTGCGGTCTGGCAAC-3' (Burnens *et al* 1996). A 30-cycle programme (94°C for 1 min, 54°C for 0.5 min and 72°C for 2 min) was used. The PCR product was electrophoresed through 1 per cent agarose gel and purified with a QIAquick-spin extraction kit (Qiagen, Chatsworth, CA, USA). The 16S rRNA and IS*200* probes were labelled with DIG-11-dUTP by using a DIG-High Prime Labeling Kit (Boehringer Mannheim GmbH, Mannheim, Germany).

Two μg of *Salmonella* DNA was digested with restriction endonucleases (New England Biolabs, Beverly, MA, USA) and electrophoresed through 0.8 per cent agarose gel in 1 x Tris-acetate-EDTA buffer. Denatured DNA was transferred to a nylon membrane (Hybond-N, Amersham International PLC, Amersham, UK) in 20 x SSC (Sambrook *et al* 1989) and fixed to the membrane using microwaves (Angeletti *et al* 1995). Hybridisation and detection were performed with the DIG Nucleic Acid Detection Kit (Boehringer Mannheim GmbH, Mannheim, Germany).

The ribo- and IS*200*-types for *S*. Infantis were named as previously (Pelkonen *et al* 1994), with additions of new profiles (II). Weak bands detected by ribotyping differed between the gels, whereas strong bands were fully reproducible. Therefore only strong bands were scored. For IS*200*, the bands detected were fully reproducible. DNA profiles differing by at least one band were assigned a profile name (capital letter) (*S*. Typhimurium) (IV). After visual analysis of the profiles, a computer program for analysis of electrophoretic patterns (GelCompar, Applied Maths, Kortrijk, Belgium) was used to generate the dendrograms (Vauterin and Vauterin 1992).

4.2.4 Other analyses (III, IV)

Polymerase chain reaction (PCR) for detection of IS*200* (*S*. Agona) (III). The primer pair 5'-CCTAACAGGCGCATACGATC-3' and 5'-ACATCTTGCGGTCTGGCAAC-3' (Burnens *et al* 1996) and a 30-cycle program (94°C for 1 min, 54°C for 0.5 min and 72°C for 2 min) was used to amplify a 557-bp PCR product of the IS*200* insertion sequence. *S.* Infantis K1469 was used as a positive control.

Testing for microbial drug resistance (*S*. Agona) (III). Agar diffusion test was performed according to the National Committee for Clinical Laboratory Standards (NCCLS) (current name Clinical and Laboratory Standards Institute, CLSI) with Oxoid disks (Oxoid, Hampshire, UK) using Mueller-Hinton agar (Becton Dickinson and Company, Cockeysville, MD, USA). The disks contained ampicillin 10 μg, cephotaxime 30 μg, chloramphenicol 30 μg, ciprofloxacin 5 μg, enrofloxacin 5 μg, streptomycin 10 μg, sulfamethoxazole-trimethoprim 25 μg and tetracyclin 30 μg.

Detection of the *spv*C virulence gene by PCR (*S*. Typhimurium DT1) (IV). PCR was performed using DyNAzyme DNA Polymerase kit (Finnzymes, Espoo, Finland) according to the manufacturer's guidelines. The reaction contained 20 pmol of the primers spvc-F ACT CCT TGC ACA ACC AAA TGC GGA and spvc-R TGT CTC TGC ATT TCG CCA CCA TCA (Chiu and Ou 1996). Bacterial suspensions were lysed at 95°C for 5 minutes and amplified over 25 cycles at 94°C for 1 min, at 56°C for 1 min and at 72°C for 1 min in a UNO II thermocycler (Biometra). The PCR product was analysed in 1.5 per cent agarose gels (Seakem LE, FMC Bioproducts, Rockland, ME, USA) with 0.5 μg ethidium bromide per ml at 4.7 V/cm for 50 min in 1 x Tris-acetate-EDTA buffer (Sambrook *et al* 1989). To confirm the presence of the serovar specific plasmid, the *spvC* gene was localized to a plasmid by hybridization. The 571 bp PCR product was amplified from a mixture of the *S*. Typhimurium isolates: DT104 (IH 59841), DT12 (IH 68594, IH 69493) and DT120 (2671), then electrophoresed through 1.5 per cent agarose gel and purified with a High Pure PCR Product Purification kit (BoehringerMannheim GmbH, Mannheim, Germany). The PCR product was labelled with DIG-11-dUTP by using a DIG-High Prime Labeling Kit (Boehringer Mannheim GmbH, Mannheim, Germany) and used as a probe for hybridization of S1-nuclease digested plasmids of *S*. Typhimurium DT1. These plasmids were transferred to nylon membranes after PFGE. Hybridization and detection were performed according to the DIG Nucleic Acid Detection Kit instructions (Boehringer Mannheim GmbH, Mannheim, Germany).

5. RESULTS

5.1 *S*. Infantis (I, II)

Analysis of the feedstuff-related outbreak in 1995 (I). DNA profiles differing by one or more DNA fragments larger than 125 kb were assigned a pulsed-field (pf) type number. When discrimination of the *Xba*I-PFGE analysed isolates was based on all visible DNA fragments, regardless of their size, the pulsed-field (pf) types could be further divided into plasmid subtypes (data not shown). The feed samples from 1995 (n=23) were all found to be of the same plasmid type. This subtype of the main pf-type pf1 (I: table 2) was designated number 39 and it was regarded as the feedstuff-related plasmid subtype. Its *Xba*I-macrorestriction profile had an intensive band of approximately 60 kb in size. The plasmid type pf1/39 was found on 74 cattle farms and in 23 feed samples in 1995, but not among samples taken from the earlier period 1992 to 1994. Similarly, plasmid type pf1/39 was not obtained in isolates from Finnish broiler chickens (300 isolates, from 1983 to 1995) nor human isolates (42 isolates, 1985 to 1994) (data not shown). Analysis with S1-nuclease revealed plasmid type pf1/39 typically to contained two plasmids of approximately 90 and 60 kb in size (I: figure 1). Moreover, 69 of the 74 farm isolates of plasmid type pf1/39 contained two plasmids of 90 and 60 kb as revealed by S1-nuclease analysis and alkaline lysis. Only five of the isolates had a different plasmid profile. They all contained the 60 kb plasmid, but other plasmids of 90, 80, 70 or 40 kb as well.

Among cattle isolates from the years 1994 and 1995, we found five plasmid subtypes of the pulsed-field type pf1 that in their *Xba*I-macrorestriction profile strongly resembled plasmid type pf1/39. No resembling plasmid subtypes were found in 1992 and 1993. The plasmid subtypes were respectively designated the numbers 43, 44, 45, 46 and 71. They all had the 60 kb band in *Xba*I-PFGE, typical of plasmid type pf1/39, in addition to other intensive bands (I: figure 1). S1-nuclease analysis revealed that they shared the 60 and 90 kb plasmids with plasmid type pf1/39, but had other plasmids of 105, 40 or 30 kb as well (I: figure 1).

The similarity of the plasmid types that resembled the feed-related plasmid type pf1/39 was studied by fingerprinting of plasmid DNA. We analysed all the strains (n = 20; 2 from 1994, 12 from 1995 and 6 from 1996) of plasmid types pf1/43, pf1/44, pf1/45, pf1/46 and pf1/71, and the feedstuff isolates of plasmid type pf1/39 (n = 23). The plasmids were isolated by alkaline lysis and the similarities of the plasmids were analysed by restriction fingerprinting using *Hind*III, *Sfi*I or *Xba*I enzymes. The best results regarding the number of easily discriminated fragments were obtained with *Hind*III (I: figure 2), but the results with the other enzymes were in agreement with those of *Hind*III (data not shown). The plasmids of the types pf1/43, pf1/44 and pf1/46 shared most of the

restriction fragments with type pf1/39. In contrast, the isolates of type pf1/45 for the years 1994, 1995 and 1996 differed from each other. The isolates for 1994 and 1996 differed from type pf1/39, whereas the isolates of plasmid type pf1/45 obtained from the farms that had received the contaminated animal feed in 1995, shared most of their restriction fragments with type pf1/39. The plasmids in type pf1/71 were of the same size as in type pf1/39, but the restriction profile differed with all three enzymes from that of type pf1/39.

The feedstuff-related plasmid type pf1/39 was detected on 12 farms in 1996, and the related plasmid types pf1/43, pf1/44, and pf1/45 were recorded for 2, 2, and 6 farms, respectively. Plasmid type pf1/45 was found on two farms in 1997 and one farm in 1998. In total, the plasmid types pf1/39, pf1/43, pf1/44 and pf1/45 accounted for 17 per cent (22/131), 11 per cent (2/18) and 5 per cent (1/22) of the pf-types in the analysed isolates from 1996, 1997 and 1998, respectively. These profiles were not detected in 1999 or 2000, but in 2001, plasmid type pf1/39 was seen on one farm (data not shown).

PFGE profiles obtained by *Xba*I (II). Among the 588 analysed cattle isolates obtained from 478 farms over the 1985 to 2003 period, for bands larger than 125 kb, 51 different *Xba*I-macrorestriction profiles (pulsed-field, pf-types) were determined (II: table 2; figure 3). The predominant macrorestriction profile in cattle was pf1, detected in 68 per cent (335/494) of the isolates. The most common pf-types pf1, pf2 and pf3 accounted for 80 per cent (396/494) of the profiles detected (II: table 2). The coefficient of similarity (F) values ranged from 0.58 to 0.95 (II: figure 3).

Among the analysed cattle farm isolates, 41, 17 and 6 plasmid subtypes of the pf-types pf1, pf2 and pf3, respectively, were determined and 78 different plasmid subtypes among the 47 other pf-types. When all bands larger than 20 kb were regarded, 142 different profiles were detected among the analysed cattle farm isolates. When the cattle transport vehicles and slaughter house hygiene samples (n= 48) were also included, and all bands larger than 20 kb, 11 additional profiles were determined; 6 of them were plasmid subtypes of the most commonly seen profile pf1. If only those bands larger than 125 kb were regarded, three additional pf-types were recorded (namely pf53, pf100 and pf140) (data not shown).

Plasmid profiles (II). The S1-nuclease analysis was carried out on 77 per cent (398/520) of the *Xba*I-PFGE analysed cattle farm isolates and 88 per cent (352/398) of the analysed isolates were found to harbour plasmids in a size range of 20 to 125 kb (data not shown). Plasmids were more common in the isolates from 1995 to 2002 than in isolates taken earlier.

Ribo- and IS*200*-types (II). Fiftyseven isolates were analysed by ribotyping and IS*200*-typing: 25 isolates from 1985 to 1987, 19 isolates from 1992 to 1995, and 13 isolates from 1999 to 2002. Among all the analysed isolates (n=57), five different ribo/IS*200*-types were detected (data not shown). The most common type was 1A, found in 89 per cent (51/57) of the isolates. The isolates obtained over the 1985 to 1987 period (n=25) had the ribo/IS*200*-types 1A (n=21), 1Q (1), 1S (1) or 1T (2) and were of the *Xba*I-macrorestriction profiles pf1 (15 isolates with ribo/IS*200*-type 1A and one isolate each with ribo/IS*200*-type 1Q or 1S), pf2 (2 isolates, 1A), pf153 (one isolate each of 1A and 1T), pf55 (1 isolate, 1T) and pf147, pf152 or pf517 (one each, all 1A). For the 1992 to 2002 period, 30 isolates had the ribo/IS*200*-type 1A and were of the pf-types pf1 (19 isolates), pf2 (4), pf35 (2), pf36, pf66, pf95, pf118 or pf192 (one each). Two isolates had the ribo/IS*200*-type 7O; both were of pf-type pf1. Ribo/IS*200*-types O, Q, S and T differ from profile A, which has bands of 0.6, 1.6 and 2.2 kb (Pelkonen *et al* 1994), by having one additional band of approximately 3 kb in size (profile O), one additional band of 3.6 kb (Q), one additional band of 1.8 kb (S), or two additional bands of 3.6 and 4 kb (profile T) (data not shown). Profile 7 differs from profile 1 by having one additional band approximately 4 kb in size (data not shown).

Follow-up on the stability of the *Xba*I-macrorestriction profiles on farms during the infection (II). From 142 farms, two or more *S*. Infantis isolates were obtained during the eradication of the infection. The typing results were analysed in three groups according to the number and timely distribution of the available isolates. Data from 26 farms were analysed in more than one group as the farms had several isolates both from the same year and different years.

From the same year, two isolates were available from 64 farms, and three or four isolates from 14 farms. The time interval between the isolates varied from 0 to 9 months. For the farms with the isolates in the same year (n=78), the same pf-type was detected in 13 (17%), and both the same pf-type and the same plasmid subtype was observed for 50 (64%) of the 78 farms. For 9 (14%) of the 64 farms that had two isolates analysed, the isolates had different pf-types. For 6 (43%) of the 14 farms with three or four isolates analysed, both the same pf-type and a different pf-type was seen. For 68 (87%) of the 78 farms the same pf-type was seen in at least two of the analysed isolates (data not shown).

From two different years, two isolates were available from 52 farms and three to five isolates from 16 farms. The time interval between isolation varied from one to three years. For the farms with isolates available from two different years (n=68), the same pf-type was seen on 25 (37%), and both the same pf-type and the same plasmid subtype was seen on 21 (27%) of the 68 farms. For 15 (29%) of the 52 farms with two isolates analysed, the isolates had different pf-types. For 5 (31%) of the 16 farms with three to five isolates analysed, both the same pf-type and a different pf-type was seen. For 52 (76%) of the 68 farms the same pf-type was seen in at least two of the analysed isolates (data not shown).

For the farms with isolates obtained over three (16 farms) or four (6 farms) different years, the same pf-type was detected on 8 (36%), and both the same pf-type and the same plasmid subtype was determined on 3 (14%) of the 22 farms. For 9 (41%) of the farms, both the same pf-type and a different pf-type was found. On 20 (91%) of the 22 farms the same pf-type was seen in at least two of the analysed isolates (data not shown).

The within farm-differences in the *Xba*I-banding patterns of successive isolates ranged from one to five bands. The difference in banding pattern for isolates from the same farm with different pf-types (data not shown) was one band for 32 per cent (10/31) and two bands for 55 per cent (17/31) of the analysed farms. For isolates from the same year, 42 per cent (5/12) had only a one-band difference and 33 per cent (4/12) had a two-band difference, and one isolate each had a three-, four-, or five-band difference. For isolates from two different years, 26 per cent (5/19) had a one-band difference, 68 per cent (13/19) a two-band difference and one isolate a three-band difference.

The regional distribution of certain PFGE profiles (II). When looking at the regional distribution of the *Xba*I-PFGE analysed isolates, which is representative of the regional distribution of the *S*. Infantis positive farms, certain macrorestriction profiles (pf-types, bands larger than 125 kb) and plasmid subtypes seem typical of certain municipalities. These profiles (pf1 and its plasmid subtypes 1/24, 1/34 and 1/37; pf2 and pf3) are common among cattle overall (data not shown). They are especially common among the analysed faecal isolates obtained over 1995 and 1996 in Kälviä (pf1/24 in all four analysed isolates), Ilmajoki and Vihanti (pf1/24; in 3 of 6 and 3 of 8 analysed isolates, respectively), Teuva (pf1/37; 5/23), Kaustinen (pf2; 6/13) and Himanka (pf3; 3/4). All municipalities mentioned, except Vihanti, are located in the former province of Vaasa. In contrast, Vihanti is in the province of Oulu.

5.2 *S*. Agona (III)

PFGE profiles obtained by *Xba*I. Digestion of all 110 isolates with *Xba*I yielded fragments ranging from 40 to 600 kilobases (kb) and yielding 39 different pf-types (III: table 2; figure 2). Among the 69 nonhuman and the 41 human isolates, 19 and 23 pf-types were detected (III: table 2). Of the 39 pf-types, 31 were recorded in only one or two isolates. The most common profile was pf1 (22 isolates) followed by pf21 (13 isolates), pf22 (9 isolates), pf5 (8 isolates) and pf19 (7 isolates). These five types represented 54 per cent (59 isolates) of the 110 isolates studied. Of these, pf5 was detected in only the human isolates, and the other common pf-types in nonhuman isolates (except for one human domestic isolate of pf1). Of the 39 pf-types, 14 were seen in isolates of foreign origin only, 5 were seen in human isolates of foreign and domestic origin, and 3 were seen

in human isolates of domestic origin only (III: table 2). The overall differences between pf1 and the 38 other pf-types obtained by *Xba*I-digestion (pf1 to 40) gave Dice similarity coefficient values larger than 0.6 (III: figure 2).

*Xba*I-profiles pf1 and pf2 were associated with the outbreak among cattle farms in 1994 and 1995, whereas profile pf39 was associated with the outbreak among humans in 1999. Only one isolate from humans had the same profile as that for the cattle outbreak but none of the cattle isolates belonged to pf39 (III: table 2). Pf1 and pf2 differed from each other only by one intensive band of approximately 100 kb, and S1-nuclease analysis revealed that pf2 contained a plasmid of the same size (III: table 3). Pf39 differed from pf1 by 9 bands (III: figure 2).

PFGE profiles obtained by *Bln*I, *Not*I and *Spe*I. Since the overall differences between the *Xba*I-profiles were not large (III: figure 2), one isolate of each *Xba*I-profile (III: table 2), and additional isolates of the most common *Xba*I-profile, pf1, were analysed by other enzymes as well (III: table 3). Among the 52 isolates analysed, digestion with *Bln*I yielded fragments of 40 to 600 kb (data not shown) and 28 different types were designated with the letters a to w. Moreover, *Not*I yielded fragments of 10 to 250 kb (data not shown) and 21 types (N1 to N21). In contrast, *Spe*I yielded fragments of 10 to 400 kb (data not shown) and 21 different types (A to T) among the 45 analysed isolates.

Although the number of profiles obtained by digestion with any of these enzymes was not as large as the number obtained by digestion with *Xba*I, most of the *Bln*I-, *Not*I- and *Spe*I-profiles were only found in one or two of the isolates analysed (III: table 3). However, the *Xba*I-profiles seen in cattle isolates obtained for the years 1994, 1995, and 1997 (profile pf1, pf2, pf3 and pf4 and profile pf21, pf22 and pf23) shared the *Spe*I-profiles A and J, respectively (III: table 3). The differences between these two *Spe*I-profiles, and those between the *Bln*I- and *Not*I-profiles (III: table 3) detected in these cattle isolates, are minor (a one to four band difference)

In the isolates obtained from the outbreak in cattle (*Xba*I-profiles pf1 and pf2) and those of the human outbreak (pf39) (III: table 3), the *Bln*I-profile a1 resembled *Bln*I-profile a though it had an additional band (data not shown). There was also a one-band-difference between the *Spe*I-profiles A and AD and *Not*I-profiles N1 and N7 (data not shown).

Plasmid profiles. S1-nuclease analysis combined with PFGE revealed that 35 per cent (38/110) of the strains harboured plasmids larger than 20 kb (data for representative strains are shown in III: table 3). Smaller plasmids with six different profiles (profiles a to f) were detected in 15 per cent (16/110) of the isolates (III: table 3).

Possession of IS*200*. The 557 bp product of IS*200* for the *S*. Agona isolates was not amplified. A strong and clear band of that size was always amplified from the control strain K1469.

Antimicrobial resistance patterns. Of the 73 domestic isolates, only one isolate from a cattle farm (strain 2476) that was recovered in 1997 showed resistance to any of the antimicrobials tested (chloramphenicol and tetracycline; III: table 3). In comparison, five of the 37 foreign isolates were resistant, with resistance to tetracycline and streptomycin being the most common (III: table 3).

Combination of profiles. When representative isolates of each of the 39 *Xba*I-profiles were analysed with other restriction enzymes (III: table 3), the relatively large number of different profiles obtained with the additional enzymes (28, 21 and 21 profiles, for *Bln*I, *Not*I and *Spe*I, respectively) supports the existence of at least minor differences between the 39 *Xba*I-profiles. The isolate representative of the human outbreak (*Xba*I-profile pf39) did not seem to be related to any of the other isolates tested, whereas two groups (groups A and J) were formed among the cattle isolates by *Spe*I digestion. Large plasmids were seen in 33 per cent (13/39) and small plasmids in 20 per cent (8/39) of the *Xba*I-profiles, but there seemed to be no relationship between the PFGE profiles and plasmid profiles. All drug-resistant strains seemed to harbour large plasmids, thereby indicating the possibility of plasmid-mediated drug resistance in the analysed isolates. Due to the large number of profiles obtained with all restriction enzymes used in the study and the lack of an obvious relationship between the PFGE and plasmid profiles, the material could not be divided into a few clear combination profile groups.

5.3 *S*. Typhimurium DT1 (IV)

PFGE profiles obtained by *Xba*I. Digestion of all 255 isolates with *Xba*I yielded 38 different *Xba*I-PFGE macrorestriction profiles differing by one or more bands. The number of fragments generated varied between 10 and 14 in the size range of 20 to 500 kb (IV: figure 1). The most common PFGE profile (profile 10) was detected in 125 of 255 isolates (49%) (IV: figure 1). This profile was recorded in human isolates obtained from the outbreaks in the 1980s and 1990s, and in sporadic cases (IV: table 2). Other common *Xba*I-profiles were 11, 20, 50 and 30, which represented 31, 17, 12 and 10 isolates, respectively (IV: figure 1). A majority of the analysed isolates, 76 per cent (195/255) had one of the five most common profiles. All other profiles were seen in less than 10 isolates each, and of the 38 *Xba*I-PFGE profiles, 27 (71%) were represented by only one or two isolates (IV: figure 1). Of the seven *Xba*I-profiles (10, 20, 21, 22, 23, 26 and 60) detected in the 20 human isolates of foreign origin, only two (21 and 22) were not seen among the domestic isolates (IV: table 2). The Dice coefficient of similarity (F) values between the 38 *Xba*I-PFGE profiles varied between 0.56 and 0.96 (IV: figure 1). Profiles 20 to 27 formed a cluster which differed from profile 10 and others at a level of 0.75.

PFGE profiles obtained by *Bln*I and *Spe*I. Isolates representing the 38 *Xba*I-PFGE profiles and different sources were analysed using *Bln*I and *Spe*I (IV: table 3). Among these 68 isolates, digestion with *Bln*I yielded 25 different PFGE profiles. The number of fragments generated varied between 7 and 10 in the size range of 40 to 600 kb. The F values between the *Bln*I-PFGE profiles ranged from 0.43 to 0.93 (data not shown). Profile 7 was the most common, detected in 49 per cent (33/68) of the analysed isolates, and among 18 different *Xba*I-profiles. Digestion with *Spe*I yielded 29 PFGE profiles. The number of fragments generated varied between 17 and 21 in the size range of 20 to 300 kb, and the F values between 0.59 and 0.97 (data not shown). Profile 1 was the most common, detected in 29 per cent (20/68) of the analysed isolates, and among seven *Xba*I-profiles.

Ribotypes and IS*200*-types. All the 52 isolates analysed (IV: table 4) belonged to only one ribotype as assessed by *Pvu*II restriction (data not shown). Twelve different IS*200*-types (A to M) were detected using *Pst*I (IV: table 4; figure 2). The number of IS*200* copies varied between 8 and 14 and only one copy in a 4.3 kb fragment was common to all *Pst*I-profiles (IV: figure 2). Profile D was the most common in both the 1980s (8/15 isolates, 53%) and 1990s (25/37 isolates, 68%), and appeared in isolates of various origins (IV: table 4). The second most common profile, A, was recorded in 17 per cent (9/52) of the isolates. It occurred only in isolates of human or cattle origin (IV: table 4). Two clusters of IS*200*-types were recorded. In dendrogram profile A was closely

related to C, whereas seven profiles, F, G, H, I, K, L, and M, were related to profile D (data not shown).

Plasmid analyses. All 255 *S*. Typhimurium DT1 isolates were analysed by S1-nuclease PFGE whereas 173 isolates were analysed by alkaline lysis. All isolates of the less common PFGE profiles detected by *Xba*I (35 profiles, 82 isolates) and 91 isolates of the three most common *Xba*I-profiles (profile 10, 62 isolates; profile 11, 17 isolates; profile 20, 12 isolates) representing different origin of isolation were analysed by alkaline lysis. All isolates harbouring plasmids larger than 20 kb, as shown by S1-nuclease, and all ribo- and IS*200*-typed isolates were analysed by PCR for the presence of the *spvC* gene (127 isolates). To confirm the presence of a *spv* plasmid, 20 *spvC* PCR positive and eight PCR negative isolates were analysed by hybridization.

S1-nuclease analysis combined with PFGE determined that 41 per cent (104/255) of the isolates harboured plasmids in a size range (50 to 211 kb) which might contain the virulence plasmid (data not shown). Of these 104 isolates, 26 per cent were positive for *spvC* by PCR (data not shown). Of the 52 ribo- and IS*200*-typed isolates, 23 did not harbour large plasmids and were negative for *spvC* by PCR (data not shown). All isolates (n=10) of the IS*200*-types A or C and 27 isolates of the *Xba*I-profiles 20, 21, 23, 24, 25, 26 or 130 had the *spvC* gene. In contrast, isolates of the IS*200*-types D, E, F, G, H, I, K, L and M did not have the *spvC* gene (data not shown). The *spvC* probe hybridized to plasmids in a size range of 90 to 108 kb in all of the *spvC* PCR positive analysed isolates (20 of 27). The probe did not hybridize to plasmid-free PCR negative isolates of IS*200*-types D (4 isolates), B (1) or I (1) or to two isolates of *Xba*I-profiles 31 and 51 (data not shown). Based on S1-nuclease analysis and *spvC* PCR in total 11 per cent (27/255) of the *S*.Typhimurium DT1 isolates had the serovar specific plasmid.

Plasmids larger than 108 kb were present in only three isolates; one each of the *Xba*I-PFGE profiles 10, 30 and 60. Only 8 per cent (10/125) of the isolates of the most common *Xba*I-PFGE profile 10 harboured plasmids larger than 20 kb. The other isolates harboured large plasmids in 72 per cent (94/130) of the isolates (data not shown).

Plasmids smaller than 20 kb were seen in 28 per cent (48/173) of the analysed isolates (data not shown). Of the analysed isolates of the most common *Xba*I-profile 10, 15 percent (9/62) contained small plasmids. Small plasmids were present in 90 percent (28/31) of the isolates of the *Xba*I-profiles 31, 40, 50, 51, 60 and 61 (data not shown).

Neither large nor small plasmids were common in the analysed human isolates of domestic or foreign origin; 29 per cent (35/120) of the domestic and 30 per cent (6/20) of the foreign isolates contained large plasmids and 26 per cent (18/70) of the domestic and 13 per cent (2/16) of the foreign isolates harboured small plasmids (data not shown). Plasmids were more common in the analysed domestic isolates from poultry, where 64 per cent (23/36) harboured large plasmids and 52 per cent (11/21) harboured small plasmids. Plasmids were also more common among the analysed domestic isolates from cattle, where 56 per cent (31/55) harboured large plasmids. Small plasmids were seen in 26 per cent (12/47) of the analysed cattle isolates (data not shown). Overall, small plasmids seemed to be less common in the isolates from the 1980s, whereas large plasmids seemed more common in these older isolates.

Combination of profiles. The compilation of the *Xba*I-, *Bln*I- and *Spe*I-PFGE results gave: 52 different combination profiles among the 68 analysed isolates, 48 profiles among the 61 domestic isolates and 7 profiles among the foreign isolates (IV: table 3). Some combination profiles, such as *Xba*I10-*Bln*I7-*Spe*I1 and *Xba*I50-*Bln*I7-*Spe*I7, were common for different infection sources. Dendrogram analysis was done separately for each of the three enzymes used for PFGE. This revealed two major clusters among the *Xba*I-, *Bln*I- and *Spe*I-PFGE profiles (IV: figure 1, data not shown). *Xba*I-profiles 20 to 27 formed one cluster and the other profiles (except profile 130) another (IV: figure 1). The *Bln*I-profiles 8, 10, 11, 18, 28 and 184, and *Spe*I-profiles 2, 6, 13, 20 and 21 also formed one cluster (data not shown).

When the PFGE results were compiled with those of IS*200*-typing and *spvC*-PCR, two clusters were obtained (IV: table 3). There was no overlapping of IS*200*-types and PFGE profiles between the clusters. They contained profiles either with or without the *spvC* gene. The isolates (n=54) of the larger cluster had IS*200*-types D, F, G, H, I, K, L or M, no *spvC* gene and one to three PFGE profiles in common. Isolates of the most common *Xba*I-profile 10 belonged to this cluster. The isolates (n=11) of the smaller cluster had IS*200*-type A or C, the *spvC* gene and *Xba*I-profile 20, 21, 23, 24, 25 or 26. These profiles were seen in human foreign and domestic isolates and in cattle. Three isolates were not placed into either of the two clusters because of their divergent IS*200*-profile (B and E) or *Xba*I-profile (130).

6. DISCUSSION

The aim of this study was to understand the molecular epidemiology of salmonella infections caused by the *Salmonella* serovars *S*. Infantis, *S*. Agona, and *S*. Typhimurium DT1. We wanted to see how the infection causing strains evolve and spread. Focus was laid on cattle isolates and the infection in Finnish cattle as *Salmonella* infection in cattle can be transmitted to humans by several routes. Some of these routes e.g. the environment are difficult to control.

6.1 Characterization of *S*. Infantis isolates (I, II)

6.1.1 Application of molecular methods

The molecular typing methods applied in the analysis of *S*. Infantis are shown in Table 2. PFGE was highly discriminatory in typing of *S*. Infantis, as has also been reported for other *Salmonella* serovars (e.g. Baquar *et al* 1994a; Powell *et al* 1994). In our study (II), a total of 51 different *Xba*I-macrorestriction profiles (i.e. bands larger than 125 kb) were detected in the 588 analysed isolates from 478 cattle farms over the years 1985 to 2003. An additional three profiles were obtained from among the 48 analysed cattle slaughterhouse and cattle transport float isolates for the period 1992 to 1995. The infection among cattle seem fairly homogenous as the predominant macrorestriction profile pf1 was found in 68 per cent of the cattle farm isolates and the most common pf-types pf1, pf2, and pf3 were detected in 80 per cent of the cattle farm isolates. Overall, 99 per cent of the analysed isolates from cattle farms had pf-types clonally related to each other (F-values above 0.7). This result supports the conclusion that the *S*. Infantis infection in cattle is endemic.

Not many studies on the molecular analyses of *S*. Infantis have been published. In a study by Merino *et al* (2003), 15 Argentinian and four Spanish strains of *S*. Infantis were analysed by *Xba*I- and *Xho*I-PFGE; all the Argentinian strains had identical profiles despite their geographical origin. The PFGE conditions differ from those we used, and no molecular weights are shown, but the Argentinian PFGE profile has all the bands typically seen in *Xba*I-PFGE of *S*. Infantis. In our analysis of isolates from Finland, we also analysed some isolates of foreign origin. There was one significant difference between these two groups of isolates: namely in the intensity of one of the bands. In the Finnish isolates, typically a band of approximately 280 kb was most intensive (number five from the top in the dominating PFGE profile pf-type pf1; Figure 1 in our study I). In contrast, in some of the isolates of foreign origin, a band of approximately 310 kb (number four from the top in a typical isolate) was most intensive. This observation is also true for the Argentinian profile. Wegener and Baggesen (1996) investigated an outbreak of human salmonellosis caused by *S*. Infantis, *Xba*I-PFGE, produced 21 different PFGE profiles from 135

analysed isolates. The banding patterns resemble those obtained from Finnish isolates, although the PFGE conditions are different. However, the exact comparison is impossible, as the Danish banding patterns are shown only as drawings, not as photographs, which would have also enabled comparisons of band intensity. Murakami *et al* (1999) analysed 110 isolates of *S*. Infantis by PFGE, 35 distinct profiles were seen but they used *Bln*I as the restriction endonuclease.

Prior to the development of PFGE, ribotyping and IS*200*-typing were considered useful for typing of isolates of *Salmonella*e (e.g. Olsen *et al* 1994b; Stanley *et al* 1994). Ribotyping and IS*200*-typing were also applicable in the analysis of the endemic *S*. Infantis infection in Finland. However, the infection has become more homogenous over time. In isolates obtained in the 1980s, up to eight different ribo/IS*200*-types were determined among domestic isolates from humans, poultry and cattle (Pelkonen *et al* 1994). The analysed cattle isolates (n=11) were all of the same ribo/IS*200*-type (1A), which later became one of the predominant ribo/IS*200*-types. In the 1990s, mainly one ribo/IS*200*-type (1A) was found among humans and cattle, and two ribo/IS*200*-types (1A and 1B) were detected among poultry (Pelkonen *et al* 1998). In this study (II), five different ribo/IS*200*-types were detected: the ribo/IS*200*-type 1A was found in 51 of the analysed 57 cattle isolates, and in isolates obtained in the 1980s, 1990s and 2000s, whereas the other ribo/IS*200*-types were only found either in samples of the 1980s (1Q, 1S, 1T) or 2000s (7O), This parallels the typing results of domestic isolates from humans and poultry from the 1980s and 1990s, in that they showed a decreased diversity over time (Pelkonen *et al* 1994, 1998).

Our studies (I, II) support the conclusion that the occurrence of plasmids is typical of our endemic *S*. Infantis infection (Pelkonen *et al* 1998). In a study by Helmuth *et al* (1985), only 12 per cent of the analysed *S*. Infantis isolates were found to harbour plasmids. In our present study (II), S1-nuclease analysis combined with PFGE showed that 88 per cent (352/398) of the analysed isolates contained plasmids. Plasmids seemed less common in the isolates of the 1980s for which the plasmid containing percentage varied between 33 and 60. In contrast, those isolates obtained from the 1990s seemed to harbour plasmids in between 57 and 100 per cent of the analysed isolates. Naturally plasmids might have been less frequent in the 1980s. However, the storage of the *S*. Infantis strains on egg agar slopes before the analysis of 1980s samples, compared to the majority of samples being stored at -70°C for strains from 1990s may be the cause for this difference. Olsen *et al* (1994a) have shown that plasmids can be lost during stab cultures. Plasmid profiles of chicken salmonella can also change rapidly (Brown *et al* 1992). Plasmids have therefore not been regarded as very reliable for long term surveillance.

A specific *Xba*I-PFGE plasmid profile not previously encountered was associated with the feed-related outbreak of *S*. Infantis among cattle in May 1995 (I). S1-nuclease analysis and *Hind*III

digestion of alkaline lysis isolated plasmids confirmed the presence of the feed-related plasmids, of which the 60 kb plasmid was visible in *Xba*I-PFGE.

6.1.2 *S*. Infantis infection on cattle farms

Certain PFGE profiles clustered with regard to certain municipalities and in some cases the epidemiological information given by the local veterinarian support our typing results (II). Confirmation was given in respect of contacts between farms, which in our analysis had the same PFGE profiles, pf1/24 and pf1/37, for the municipalities of Kälviä and Teuva, respectively. Additional data was not available for all municipalities regarding the exact location of the individual farms and any possible contacts between them.

The ribo/IS*200*-types 1A and 1O and the most common pf-types seen among the analysed cattle isolates can also be seen among domestic isolates of poultry and humans (Lindqvist *et al*). Ribo/IS*200*-type 1A, which is typical of the endemic *S*. Infantis infection was also determined among human isolates of foreign origin and among foreign animal isolates from 1993. However, these foreign ribo/IS*200*-type 1A animal isolates have pf-types not yet detected among Finnish cattle isolates. On the other hand, pf-types determined among Finnish cattle isolates can also be detected among foreign isolates of both human and animal origin of the 1990s. Even so, the ribo/IS*200*-types of those foreign isolates differ from those of the Finnish cattle isolates. Animal isolates of foreign origin of the 1980s did not share profiles with the Finnish cattle isolates though they did share profiles with domestic human and poultry isolates (Pelkonen *et al* 1998). As the Finnish cattle isolates have both the ribo/IS*200*-type and the PFGE profile in common only with other isolates of domestic origin further suggests the spread of an endemic infection. Moreover, it shows the importance of using more than one typing method when evaluating possible transmission routes of infection if detailed epidemiological data are not available.

Not many studies are published on the stability of PFGE profiles. Nevertheless, in those published, at least some of the profiles seem to be relatively stable. A study by Thong *et al* (1996) indicated that certain PFGE profiles of *S.* Typhi of Santiago, Chile, persisted over a time period of 11 years. The PFGE profiles remained almost stable in strains of *S.* Berta obtained during a nationwide outbreak in Denmark from 1984 to 1992 (Olsen *et al* 1996a). Two predominant PFGE profiles of *S.* Typhi in Papua New Guinea have remained dominant and stable over the period from 1992 to 1999 (Thong *et al* 2002). In a study by Refsum *et al* (2002), PFGE patterns of *S*. Typhimurium isolates from passerines were found to be stable over time periods of 20 to 30 years. In our study (II), the predominant *S*. Infantis pf-type pf1 was detected in the 1980s in addition to every year during the 1992-2001 period. In 2002, there was only one *S*. Infantis positive farm, but in 2003, pf-

type pf1 was detected again. The second most common pf-type pf2 was also found in cattle in both the 1980s and the 1990s. Pf-type pf1 was the only type associated with the endemic bovine infection both at the start of our analysis period in 1985, and as the infection seemed to fade out in 2003. During this time period, 50 other pf-types appeared and vanished. Thus there is no apparent trend towards increasing genetic diversity of PFGE profiles over this time period.

The national control programme of salmonella in cattle requires bacteriological monitoring of infected herds by faecal sampling. Cultures received from successive samplings enabled us to follow the PFGE profiles on a total of 142 farms (II). The profiles seemed fairly stable. Changes in *Xba*I-banding patterns were found in 22 per cent of the herds. Usually this manifested as a one or two band difference. The pf-type was the same in at least two of the isolates analysed from each farm: in 87 per cent of the farms in the same year (68 farms of 78), 76 per cent of farms for two different years (52/68) and 91 per cent for three or four years (20/22). Nevertheless, as minor changes in PFGE banding patterns seem to occur relatively frequently during long-lasting infections, it is advisable to test several isolates from a herd in outbreak investigations.

After the feedborne *S*. Infantis outbreak in 1995, the 60 kb plasmid associated with the outbreak strain was found to be stable on the infected farms for up to 15 months (I). However, the material was very limited. The feedborne plasmid of 60 kb was stable in the 12 analysed isolates representing 11 farms though the plasmid subtype changed more readily (7 isolates). Even so the pf-type pf1 remained unchanged in 10 of the isolates. In the two where it had changed, the change could be explained by a point mutation. The feed-related genotype and the related plasmid subtypes seemed to disappear relatively rapidly after the outbreak. In 1997, only two remained, and in 1998, only one farm had any of these types. Similarly, the pf-types were not found in 1999 or in 2000. However, in 2001 profile pf1/39 was detected on one farm (II). *S*. Infantis infection had not been detected on this farm in the 1990s. It is impossible to say, whether the particular infection faded out, or whether the genotype lost its specific plasmid. Without the 60 kb plasmid, the appearance in *Xba*I-PFGE would not be distinguishable from the dominating PFGE profile pf1 in any way. Furthermore, the trend in the number of cattle farms infected with *Salmonella*, and *S*. Infantis in particular has also been rapidly decreasing since 1997 (Figure 1).

The increase in the number of *S*. Infantis positive farms in 1995 (Figure 1) was due to both increased sampling, which detected subclinical infections, and to the spread of the infection via feed. In the latter case the spread was both mediated directly and indirectly as secondary infections to the feedborne infection. A peak in the number of non-Infantis salmonella farms in 1996 was as a result of the increased monitoring for bovine salmonella. This policy was carried out both as a part of the national *Salmonella* control programme and as the industry's own more

rigorous health standard checks. The national control programme of salmonella in cattle requires bacteriological monitoring of infected herds by faecal sampling of all animals on the farm. Culling of infected animals is recommended for the eradication of the infection. Epidemiological investigations are carried out on the farms to identify routes and sources of infection and manifestations of disease. The infection is often subclinical. Together with the local veterinarians, control programmes for the individual farms are made. These include improvements in production hygiene, efficient in-house cleaning and disinfection, and proper storage and handling of slurry and contaminated water. Emphasis is put on proper rodent control as well as controlling wild birds, insects, cats and dogs. No animals are allowed to have access to the feed stores. The cowshed is divided into 'clean' (feed) and 'dirty' (manure) areas. The equipment used at the farm is equally divided, so as to avoid cross-contamination. There are also footbaths with active disinfectants provided at the entry to the cowsheds. The movements are planned so that the 'clean' and 'dirty' routes will not cross. If that is not possible, footbaths are placed at the crossings. The farms maintain a closed-herd policy. Animals are not sold until the farm is free of *Salmonella* (two negative faecal cultures taken one month apart). Visitors, such as veterinarians, are provided with clean protective clothing and disinfected boots. Once the infection is eradicated, it is important to continue with the good hygiene routines. Only feedingstuffs that are free of *Salmonella* are purchased. No unnecessary visitors are allowed. Any replacement animals have to be free of *Salmonella*, and the cattle transportation vehicles cleaned between animal transports. The rapid elimination of the *S*. Infantis infection in addition to other bovine salmonella infections in the late 1990s indicates that by correct control measures the infection can be eradicated or kept at an acceptable level.

6.2 Characterization of *Salmonella* Agona isolates (III)

6.2.1 Application of molecular methods

The molecular typing methods applied in the analysis of *S*. Agona are shown in Table 2. PFGE proved most useful for typing of *S*. Agona. The isolates did not harbour any IS*200* element, as has also previously been reported (Lam and Roth 1983; Gibert *et al* 1990), rendering this method unapplicable. *S*. Agona is also unlikely to be subdivided by ribotyping (Threlfall *et al* 1996). With few exceptions, the analysed isolates were sensitive to all antimicrobials tested, thus antibiogrammes did not provide useful epidemiological markers.

We saw a total of 39 different PFGE profiles by *Xba*I-digestion of 110 strains of *S*. Agona. The overall differences between the pf-types were small and most pf-types were encountered in only one or two isolates. The importance of the ability to differentiate the isolates into that many subgroups can be questioned, and too many conclusions based on the results of one restriction enzyme only should not be drawn. Analysis using the additional enzymes *Bln*I, *Spe*I and *Not*I, support our findings even though the pf-types by *Xba*I were very similar. Thus the differences seen cannot be disregarded. In contrast, the situation was the opposite only for the pf-types pf1, pf2, pf3, pf4, pf21, pf22, and pf23, where analysis with other enzymes in addition to *Xba*I supported our conclusions that these pf-types are closely related.

Threlfall *et al* (1996) described the application of *Xba*I-PFGE to characterizing an international outbreak strain of *S*. Agona. Different conditions under which PFGE was carried out prevent direct comparison between data of our studies. Nevertheless the *S*. Agona chromosome seems to have many conserved restriction sites for the restriction enzyme *Xba*I since many fragments are common between our different PFGE profiles. Double bands, i.e. bands containing two fragments of roughly the same size but visible as one thick band, are commonly produced by the action of *Xba*I on *S*. Agona DNA. Similar observations can be made when comparing our results with the results of a study by Rabsch *et al* (2005). In their study strains of *S*. Agona from 1969 to 2003 were analysed using *Xba*I-, *Bln*I-, and *Spe*I-PFGE. Moreover, in a study by Nesse *et al* (2003), 13 different PFGE profiles were obtained when 51 isolates of *S*. Agona from fish feed factories and other sources in Norway were analysed by *Xba*I-PFGE. Some of their dendrogram profiles resemble those obtained in our analysis, but comparison by just the naked eye alone cannot be anything more than indicative. In another study by Nesse *et al* (2005), 27 isolates of *S*. Agona obtained from various sources in Norway were analysed by *Xba*I-PFGE. Unfortunately, direct comparison between the dendrograms of the 12 different PFGE profiles they obtained and our dendrogram profiles is not possible as no molecular weights are shown in their dendrogram. In a rough comparison, none of their profiles seem identical to any of our 39 profiles, but many bands seem common between the profiles in both studies. In an analysis of 59 isolates of S. Agona from Texas, USA (Taylor et al 1998), the isolates were determined to be indistinguishable if they had the same number of restriction bands and the bands were of the same size. Isolates were considered similar if they had a difference in only one band. The outbreak pattern was detected in 18 of the 59 patients. A similar pattern was detected in 8 patients, whose history could not be connected with that of the patients with the outbreak pattern. This data suggested that even a single band size difference may be enough to exclude a patient from an outbreak investigation. Michael *et al* (2006) used both *Xba*I-PFGE and *Bln*I-PFGE in their analyses of 45 *S*. Agona isolates obtained from slaughter pigs in Brazil. *Xba*I-PFGE yielded 10 profiles whereas *Bln*I yielded

seven profiles. Some of their profiles resemble those we obtained in our analysis, but again, different conditions under which the PFGE was carried out make the comparison limited.

Plasmid analysis did not provide any additional information in our analysis of serovar Agona. However, analysis of large plasmids using S1-nuclease was helpful in interpreting the *Xba*I-PFGE profiles, as some additional bands in the profiles were related to plasmids of that particular size. In addition, the presence of plasmids larger than 20 kb complicated the interpretation of banding patterns where the generated fragments were small, such as after digestion with *Not*I and *Spe*I for which the fragment size ranged from approximately 10 kb upwards.

6.2.2 Outbreak analyses

Salmonella Agona caused two small outbreaks among cattle, in the 1994-1995 and in 1997 periods, and a relatively large outbreak among humans in 1999.

Four *Xba*I-profiles (pf1, pf2, pf3, and pf4) were associated with *S*. Agona infection on cattle farms in 1994-1995. The profiles pf1 and pf2 were regarded as outbreak profiles. None of these profiles were previously seen among analysed domestic or foreign isolates. However, as *S*. Agona was seldom encountered in Finland before 1994, the number of analysed isolates from the years prior to the outbreak was very small. The outbreak profile pf1 was found in the area of the outbreak in north-western Finland in slaughterhouse hygiene samples in 1996 and 1997. A further finding was in fur animals in 1996. Fur animals are often fed slaughterhouse waste, and although it is usually treated with heat or formic acid to avoid contamination, *Salmonella* is sometimes isolated in samples obtained from fur animals. Only one domestic human isolate of pf1 was detected, and it was in 1997, long after the outbreak among cattle.

Three *Xba*I-profiles (pf21, pf22, and pf23) seemed to be related to a small outbreak among cattle in Finland in 1997. The differences of three to six bands between these profiles and pf1 can be explained by point mutations, and suggests that the outbreaks in 1994-1995 and 1997 were possibly caused by strains that can be considered related (Maslow *et al* 1993; Tenover *et al* 1995). These three pf-types (pf21, pf22, and pf23) had the same *Spe*I-profile. Pf22 and pf23 also shared the *Bln*I- and the *Not*I-profile.

The outbreak in 1999 occurred in the region of Vaasa relatively close to the cattle farm outbreak in 1994-1995 and involved more than 50 human cases. Epidemiological investigations were carried out by the local authorities, but the source of the outbreak remained unknown. There seemed to be

a connection with fur animals, which were fed waste picked up from the restaurant. One would therefore have thought it likely to detect the profile pf1 in the human outbreak isolates instead of a new profile, pf39. However, there is at least a seven band difference between pf1 and pf39 thereby indicating unrelated profiles (Maslow *et al* 1993; Tenover *et al* 1995). Pf1 was still found in slaughterhouse isolates obtained in south-eastern Finland in 1999. However, based on the molecular typing it is much more likely that the human outbreak in 1999 was not related to traces of the cattle farm outbreak in 1994-1995. Without the detailed typing results, an infection route from cattle (1994 onwards) to fur animals (1996 onwards) and finally to humans (1999) would have been easily accepted as the cause of the human epidemic.

None of the isolates of foreign origin had the *Xba*I-profiles pf1, pf2, pf3, pf4, pf21, pf22 or pf23, which were seen in domestic cattle isolates. Neither did they have the profile pf39, which was related to the restaurant outbreak among humans in 1999. However, as no recent foreign isolates were available, a foreign source for the outbreak in 1999 cannot be disregarded. The many *Xba*I-profiles detected among the 30 human isolates of foreign origin (21 profiles) may reflect the heterogeneity of the worldwide *S*. Agona infection. Five of the profiles were also found among human isolates of domestic origin. It cannot be excluded that some isolates were misclassified as foreign, or some infections classified as domestic have in fact a foreign origin.

The incidence of *S*. Agona on cattle farms peaked in 1997 and declined to a baseline of only one or two ‘positives’ a year by 2000. After 2000, no farms tested positive for *S*. Agona were detected (National Veterinary and Food Research Institute 1985-2005). Based on comparing the typing results of subsequent isolates to those of the outbreak of the 1994-1995 period, we suggest that an infection by a new strain caused the outbreak in cattle in 1994-1995. Moreover, we consider it highly likely, that a closely related strain caused the infection among cattle in 1997. The outbreaks among cattle were not associated with the outbreak among humans in 1999. They do not seem to have affected the annual level of domestic *S*. Agona infection among humans in Finland (National Public Health Institute 1985-1998; Ministry of Agriculture and Forestry 2000; Finnish Food Safety Authority Evira 2006).

6.3 Characterization of *S*. Typhimurium DT1 (IV)

6.3.1 Application of molecular methods

The molecular typing methods applied in the analysis of *S*. Typhimurium DT1 are shown in Table 2. PFGE proved most useful and specific also for *S*. Typhimurium DT1. In total 38 different *Xba*I-PFGE profiles were detected among 255 isolates of *S*. Typhimurium DT1 from 1984 to 1999, and 36 profiles were associated with infections of domestic origin. Of the seven profiles detected among isolates of foreign origin, five were also found among domestic isolates. The predominant profile (profile 10) was frequent among isolates of both human and animal origin in the 1980s and 1990s. Five different *Xba*I-profiles were detected in the 17 analysed isolates for the 2002-2004 period. These were analysed according to the standardized PulseNet PFGE Protocol (www.cdc.gov/pulsenet). The predominant profile (profile 10) was found on three of the nine positive cattle farms. Profile 20, previously only seen in human and cattle isolates, was still seen on four cattle farms but also in all isolates from pigs (n=4) and turkeys (n=3) (unpublished results).

Molecular subtyping by only *Xba*I-PFGE is not specific enough to discriminate in our endemic *S*. Typhimurium DT1 infection. Therefore it is advisable to use a combination of restriction enzymes. When the combination of the results of the three restriction enzymes (*Xba*I, *Bln*I and *Spe*I) and PFGE was used, 54 profiles were detected. As result of our work a standardized protocol for the analysis of *S*. Typhimurium DT1 can be used, which involves a combination of *Xba*I-, *Bln*I- and *Spe*I-PFGE. A new molecular method, the multiple-locus variable-number tandem-repeats analysis (MLVA) (Lindstedt *et al* 2003, 2004), was used in the analysis of isolates of our endemic highly homogenous *S*. Typhimurium DT1 infection for the time periods 1983-1995 (n=52) and 2003-2007 (n=28) (Heinikainen S, Pelkonen S). Twenty-three different profiles were detected: 16 profiles in 1983-1995 and 11 profiles in 2003-2007. In 1983-1995, 60 per cent of the analysed isolates had the same profile whereas only 14 per cent of the isolates were of the same profile in 2003-2007. Overall there was more diversity in the MLVA profile in the more recent isolates. Four profiles were common for the two time periods, and there was no clustering regarding the time or origin of isolates. For the isolates obtained from the 1983-1995 period, PFGE seemed more discriminatory than MLVA. Even so, the two major clusters that were formed by combining the results of PFGE and IS*200*-profiling could also be detected by MLVA typing. These results suggest that MLVA could be used in outbreak investigations and also in long term surveillance.

In a study on *S*. Typhimurium DTs 1, 9, 126, and 135 (Jeoffreys *et al* 2001), *Xba*I-PFGE had limited discriminatory power both between and within phage types. Other published studies on

molecular analyses of *S*. Typhimurium DT1 are scant, but for other phage types of *S*. Typhimurium, *Xba*I-PFGE on its own has not always been very discriminatory. In a study by Olsen *et al* (1997), *Xba*I-PFGE detected only one PFGE profile for isolates of *S*. Typhimurium phage types DT110, DT120 and DT135, and four PFGE profiles for isolates of DT49. However, the number of analysed isolates was quite limited; 18 (DT49), 10 (DT110), 5 (DT120) and 7 (DT135). When *Xba*I-PFGE, among other molecular methods, was used for the characterization of an outbreak of a strain of multiresistant *S*. Typhimurium DT104 (Lawson *et al* 2004), it did not discriminate between the outbreak strain and most other multiresistant DT104 isolates. These results, together with the results of our study, indicate that *Xba*I-PFGE on its own may not be discriminatory enough within one specific phage type of *S*. Typhimurium.

Rivoal *et al* (2006) compared the discriminatory power of *Xba*I-, *Spe*I-, and *Bln*I-PFGE for 399 isolates of *S*. Typhimurium (no data on phage types). Not all isolates were typable by the restriction endonucleases, but 65 (*Xba*I), 73 (*Spe*I) and 95 (*Bln*I) different profiles were obtained and 143 combination profiles could be defined. As *Bln*I was more discriminatory than the other two enzymes, and the patterns obtained were more easily interpreted than those generated by *Spe*I, a combination of *Xba*I- and *Bln*I-PFGE is recommended. Heir *et al* (2002) analysed 102 epidemiologically unrelated isolates of *S*. Typhimurium by *Xba*I-PFGE and obtained 46 distinct patterns. However, for a majority of the isolates the phage types were not determined. In a study by Foley *et al* (2006), 86 *Xba*I-PFGE patterns were generated among 128 isolates of *S*. Typhimurium, but again no data on phage types were obtained. Phage typing was considered vital by Gatto *et al* (2006) in cases of outbreak investigations. In their study, 28 different *Xba*I-PFGE profiles were identified among 1060 strains of *S*. Typhimurium DT104. In another analysis of *S*. Typhimurium DT104 (Malorny *et al* 2001), 32 strains isolated from healthy German pigs were analysed by *Xba*I-, *Bln*I- and *Spe*I-PFGE. The study found that *Bln*I and *Spe*I were equally discriminatory yielding 7 profiles each whereas *Xba*I yielded 4 different profiles. Not surprisingly, a combination of the PFGE profiles obtained with the three restriction endonucleases was even more discriminatory; 11 combination profiles were obtained. Kariuki *et al* (1999) analysed 64 isolates encompassing 11 definitive phage types of *S*. Typhimurium by *Xba*I- and *Spe*I-PFGE. Eight PFGE clusters were detected, but there was no consistent pattern of association between the phage type of the isolate and a PFGE cluster. In a study by Woo and Lee (2006), phage types could not be determined for the 118 isolates of *S*. Typhimurium. However, they found *Xba*I-PFGE to be more suitable and cost-effective than *Bln*I- and *Spe*I-PFGE. Furthermore, *Xba*I analysis of 38 isolates of *S*. Typhimurium yielded 11 different PFGE profiles. No data on *Bln*I- and *Spe*I-PFGE were given.

Ribotyping was not discriminatory at all in our study (IV) of *S*. Typhimurium DT1 as all the 52 analysed isolates were of the same ribotype in which only the strong and fully reproducible bands

were scored. In a study by Jeoffreys *et al* (2001), two different ribotypes were detected among 38 isolates of DT1. Millemann *et al* (1995) and Nastasi *et al* (1993) obtained several different ribotypes for *S*. Typhimurium, but no data was given on the phage types of their isolates. When reviewing the literature, this quite often seems to be the case.

Among the 52 isolates of *S*. Typhimurium DT1 we analysed by IS*200*-typing, 12 different IS*200*-types were detected by *Pst*I digestion. All except ten isolates were of the type A or D. Type D seemed to be typical of our endemic isolates from humans and animals in the 1980s and 1990s. It was seen in 64 per cent (7/11) of the analysed isolates from cattle. Type A was also seen in two cattle farm isolates in addition to human isolates of both domestic and foreign origin. In a study of 38 strains of *S*. Typhimurium DT1, which initially were assumed to be similar because they appeared to be epidemiologically related, three different IS*200*-types by *Pvu*II digestion were detected (Jeoffreys *et al* 2001).

Only one band (4.3 kb) was conserved among our 52 isolates of *S*. Typhimurium DT1 that were restricted by *Pst*I for IS*200*-analysis. The other bands we observed of approximately 24, 22, 19, 9.4, 4.7, 2.6 and 2 kb could correspond to the conserved *Pst*I bands reported in studies by Baquar *et al* (1993), Stanley *et al* (1993), and Millemann *et al* (1995). This suggests that there are certain conserved *Pst*I bands among isolates of *S*. Typhimurium regardless of the phage type.

Only 41 per cent of the analysed 255 isolates of *S*. Typhimurium DT1 contained plasmids in the size range of the serovar specific virulence plasmid, and only 11 per cent of the 255 isolates possessed the *spvC* gene on a plasmid. One would have expected a higher proportion of plasmid carrying *S*. Typhimurium, as this serotype has been associated with a serotype-specific virulence plasmid of a distinct molecular weight. Helmuth *et al* (1985) showed that 88 per cent of the 60 analysed isolates of *S*. Typhimurium carried a plasmid of approximately 60 MDa and only one of the strains carried no plasmid at all. Further analysis of the 60 MDa plasmid revealed an increased virulence in plasmid carrying strains. Woodward *et al* (1989) analysed the distribution of virulence plasmids within *Salmonella*e. They found that all three isolates of *S*. Typhimurium DT1 showed homology to the 10 MDa virulence region. The natural *S*. Typhimurium population can exist as high-virulence or low-virulence subclones (Helmuth *et al* 1985). In our study, the widely distributed *Xba*I-profiles associated with IS*200*-type cluster D, F, G, H, I, K, L, and M do not carry the *spvC* plasmid. These endemic strains might be of lower virulence. The low virulence might be associated with the persistence and endemic nature of the *S*. Typhimurium DT1 infection in Finland. The isolates of seven *Xba*I-profiles (20, 21, 23, 24, 25, 26 and 130) contained the *spvC* plasmid. These profiles resembled each other in *Xba*I-PFGE and in IS*200*-types (A, C).

6.3.2 *S*. Typhimurium DT1 infection

The analyses of *S*. Typhimurium DT1 isolates with both PFGE and IS*200*-typing suggest that our endemic infection is fairly homogeneous; *Xba*I-PFGE profile 10 and IS*200*-type D seem typical for our domestic isolates since the 1980s. This combination is considered to represent the major of the two most prominant clonal lineages of *S*. Typhimurium DT1 seen in Finland (*Xba*I-PFGE profile 10; IS*200*-type cluster, D, F, G, H, I, K, L, and M; no virulence plasmid). The other clonal lineage, which carries the *spvC* plasmid, is represented by the *Xba*I-PFGE profiles 20, 21, 23, 24, 25, 26, and 130 along with the IS*200*-types A and C. The IS*200*-type A (with *Xba*I-profiles 20, 21, 23, or 26) was fairly common among the analysed human isolates of foreign origin. It was also found among Finnish cattle isolates where human carriers who had visited abroad might have introduced it. In the 2000s, *Xba*I-PFGE profile 20, which in earlier years had been detected in human isolates (both domestic and foreign) and cattle isolates, was now also seen in isolates obtained from poultry and pigs (unpublished results). These were all regionally clustered. No human isolates were available from the same time period. As the number of analysed isolates is small (poultry isolates from 1981 to 1999: 36 isolates; from 2002-2004: 3 isolates), the conclusions that can be drawn are very limited. However, it appears as if this 'foreign' type of DT1 first infected the cattle farms and then later some poultry flocks.

Outbreaks of *S*. Typhimurium DT1 among humans in Finland involved consuming unpasteurized milk in the 1960s and 1970s. In the 1980s, human carriers of *S*. Typhimurium DT1 seemed to spread the infection to others. An increase in cases of domestic human salmonellosis caused by *S*. Typhimurium DT1 has been seen in late summers and autumns since the 1960s (National Public Health Institute 1965-1994) (Ministry of Agriculture and Forestry 2000). The sources of human infections, especially the sporadic events, are unknown in most cases. The most common of the endemic profiles, *Xba*I-PFGE profile 10, was found in analysed isolates from hedgehogs and wild birds. These might act as important reservoirs, by maintaining a certain baseline level of *S*. Typhimurium DT1 in the environment, and consequently be the possible sources for human infections. A study by Refsum *et al* (2002) suggested that wild passerine birds were an important source of human serovar *S*. Typhimurium infections in Norway. Comparison of Refsum *et al* (2002) and our *Xba*I-PFGE data is inconclusive. However, their subclusters A1 (pigeon) and B1 (gull) resembles our *Xba*I-profile 20, found mainly in human isolates of foreign origin and in cattle farm isolates. Their subcluster B3 (small passerine) resembles our *Xba*I-profile 27, seen in an isolate obtained from a horse in 1994. None of the five most prevalent subclusters reported in the publication, namely A1, A2, B1, B3 and G1, resembles our *Xba*I-profile 10. In Finland, mainly other phage types of *S*. Typhimurium than DT1 are found in birds, whereas DT1 is associated with hedgehogs (National Veterinary and Food Research Institute 1965-2005). Refsum *et al* (2002) also

suggested that hedgehogs constitute the primary reservoir of certain *S*. Typhimurium clones (J1 and K). As these profiles are not shown in their publication, comparison with our results is unfortunately impossible. Furthermore, most of the analysed isolates in their study lack phage typing results. As Norway is fairly close to Finland, both geographically and culturally, a comparison would have been interesting. *S*. Typhimurium is also the dominant serovar for salmonellosis acquired in Norway, and considered endemic in that country (Heir *et al* 2002).

Sporadic cases of *S*. Typhimurium DT1 are still seen among humans. The source is often unknown, though the source for an outbreak can often be established by epidemiological investigations. An outbreak of *S*. Typhimurium DT1 whose source was in homemade cheese in 1999 (Skogberg *et al* 1999) was analysed by *Xba*I-PFGE. Profile 10 was seen in isolates from this outbreak and in isolates from outbreaks of the 1980s and 1990s. However, the immediate source for the sporadic cases seen in Finland cannot be determined as the *Xba*I-PFGE profile 10, IS*200*-type D is predominant and widely distributed. This would also be the case, if no food isolates were available as might occur in outbreak investigations. Without determining the source, focused control measures cannot be implemented. When *S*. Infantis appeared in chickens in Finland in 1971, hundreds of sporadic cases were observed in humans (Simula and Jahkola 1972). However, measures to control the infection in broiler chickens were taken and have been evaluated (Rantala 1976; Seuna 1981). A national *Salmonella* control programme was started in broiler production, including in-house control measures. *Salmonella* certifications were demanded for poultry meat entering Finland. Restrictive orders were given to breeder-flocks detected positive for *Salmonella*. These orders resulted in slaughtering of the birds. *Salmonella*-positive flocks were slaughtered at the end of the day, and the meat was heat-treated. Epidemiological investigations were carried out in order to identify the source of infection. The infected poultry farm was cleaned and disinfected. Before new birds were taken to the farm after cleaning the farm, sampling results had to be negative for *Salmonella*. In addition, consumers were advised on how to hygienically prepare broiler chickens in the kitchen. As a result, the prevalence of salmonella in broilers has dropped, and the spread of *S*. Infantis infection from broilers to humans has been greatly reduced (Pelkonen *et al* 1998). Similarly, determining possible reservoirs for *S*. Typhimurium DT1 would enable focused control measures, which in turn would diminish the number of cases of *S*. Typhimurium DT1 in the future.

Table 2. The applicability of the molecular typing methods used in the analysis of *Salmonella* serovars Infantis (I, II), Agona (III), and Typhimurium DT1 (IV).

Typing method	**General comments on the application of the method**	***S*. Infantis (I, II)**	***S*. Agona (III)**	***S*. Typhimurium DT1 (IV)**
plasmid profile (alkaline lysis or S1-nuclease combined with PFGE) (presence of *spv*C virulence gene on plasmid)	Poor discriminatory power for strains that lack plasmids or have only one or two of them. Suitable for analysis of recent events. Not for longterm surveillance, as plasmids can be lost, or spread rapidly.	Plasmids seem typical of our endemic infection. A specific *Xba*I-PFGE plasmid profile was associated with the feed-related outbreak among cattle in May 1995.	Plasmids were not common in the analysed isolates. Analysis of large plasmids by S1-nuclease was helpful in interpreting the *Xba*I-PFGE profiles, as some additional bands in the profiles were related to plasmids of that particular size.	Plasmids were less frequent than would be expected according to the literature. Only 11 per cent of the isolates possessed the *spv*C gene.
plasmid fingerprinting	Used for discrimination between plasmids of similar molecular mass. Used to determine the degree of molecular relatedness between plasmids of different size. May be difficult to interpret results if a strain carries multiple plasmids.	Confirmed the presence of the feed-related plasmids, of which the 60 kb plasmid was visble in *Xba*I-PFGE. Differentiated between plasmids of the same size.	Not applied.	Not applied.

(Table 2, continued)

Typing method	**General comments on the application of the method**	***S*. Infantis (I, II)**	***S*. Agona (III)**	***S*. Typhimurium DT1 (IV)**
ribotyping	Fingerprints generally easy to interpret and reproduce. Limited discriminatory power, which can be increased by combining with other methods. Can be suitable for discrimination both within serotype and phage type when combined with other methods.	Not discriminatory. As the infection has become more homogenous over time, a majority of the analysed isolates had the same ribotype.	According to the literature not likely to be subdivided by ribotyping, therefore ribotyping not applied.	Not discriminatory for the analysed isolates.
IS*200*-typing	Fingerprints generally easy to interpret and reproduce. More valuable for differentiation within serotypes where phage typing schemes do not exist. Often used in taxonomic studies of relationships between and within serovars. IS*200*-elements are not possessed by all serotypes or phage types.	Not discriminatory. As the infection has become more homogenous over time, a majority of the analysed isolates had the same IS*200*-type.	Does not carry the IS*200*-element, therefore method not applicable. (absence of the IS*200*-element in isolates of *S*. Agona determined by PCR analysis)	Not as discriminatory as PFGE, but gave additional information. Two clonal groups could be defined by combining IS*200*-typing results with results from PFGE and plasmid analysis.

(Table 2, continued)

Typing method	**General comments on the application of the method**	***S*. Infantis (I, II)**	***S*. Agona (III)**	***S*. Typhimurium DT1 (IV)**
PFGE	Fingerprints easy to reproduce. Highly discriminatory for *Salmonella*. Suitable for discrimination both within serotype and phage type. Standardized protocol for PulseNet US and European Enternet, *Xba*I-PFGE profiles stored in central database.	*Xba*I-PFGE highly discriminatory. Superior to the other methods applied in this study.	*Xba*I-PFGE highly discriminatory, overall differences between profiles small, most profiles encountered in only one or two isolates. Analyses with the additional enzymes *Bln*I, *Not*I, *Spe*I were helpful, especially in analysing the cattle farm outbreaks.	*Xba*I-PFGE discriminatory, but one predominant profile. Analyses with additional enzymes (*Bln*I, *Spe*I) needed in case of outbreak investigations.

6.4 Molecular typing methods in the analysis of *Salmonella*e

When deciding upon the best typing strategy, the reasons for typing and the serotype of the isolates have a considerable influence. A combination of typing methods is advisable for discrimination between strains. In addition, as the typing methods measure the genetic changes that occur, some methods are more applicable for outbreak investigations than for longterm surveillance (e.g. plasmid analysis). Some typing methods are also very specific, and the interpretation of a single band difference is not always easy.
Nevertheless, the *Xba*I-PFGE method has been successfully applied for many serovars of *Salmonella*e e.g.: *S*. Agona (Threlfall *et al* 1996), *S*. Brandenburg (Baquar *et al* 1994), and *S*. Javiana (Lee *et al* 1998). The same method has also been used within phage type e.g.: *S*. Enteritidis (Powell *et al* 1994), and *S*. Typhi (Nair *et al* 1994). PFGE was also found to be the most useful for all three serovars analysed in this study (I-IV). International publications on molecular analyses of *S*. Typhimurium DT1 are scant, but for other phage types of *S*. Typhimurium, *Xba*I-PFGE on its own has not always been very discriminatory.

In a study by Murase *et al* (1996), 28 isolates of *S*. Enteritidis obtained from 19 patients in a food poisoning outbreak were analysed by *Xba*I- and *Bln*I-PFGE. Variations in PFGE patterns were observed for *Bln*I but not for *Xba*I; hence the choice of restriction endonuclease seems critical. For example, Rivoal *et al* (2006) have also shown *Bln*I to be very specific, but *Bln*I is much more expensive than *Xba*I. One factor affecting the choice of restriction endonuclease is the number of isolates to be analysed, as the costs are considerable. A balance has to be found between a restriction endonuclease having enough discrimination between the isolates without being too costly. If the number of isolates to be analysed is large, often only one restriction endonuclease is used. Therefore, despite some reports on *Xba*I not being discriminatory, it is still the restriction endonuclease chosen for PulseNet US, an American collaboration for surveillance of foodborne pathoges (http:/www.cdc.gov.pulsenet), in addition to that used for PulseNet Europe.

The use of Dice similarity coefficient assumes that bands of identical size are genetically homologous. However, fragments that migrate the same distance do not always contain homologous genetic material. Moreover, PFGE often fails to resolve bands of nearly identical size. In a study on *Escherichia coli* O157:H7, Davis *et al* (2004) recommended the use of six or more restriction enzymes for PFGE to provide a reasonable estimate of genetic relatedness. They also stated that visual evaluation of gel images is essential, as software cannot be relied upon in assessing the presence or absence of bands. Brown *et al* (2006) suggested, that in addition to the “Gold Standard” PFGE typing, plasmid typing should be considered, as it might simplify the interpretation of PFGE results. Overall, the presence of plasmids larger than 20 kb makes the

interpretation of banding patterns complicated, especially for restriction endonucleases which generate fragments that are small, e.g. after digestion by *Not*I and *Spe*I.

Murase *et al* (1996) suggest that examining multiple colonies on plates by PFGE diminishes the risk of misinterpretation arising from point mutations of chromosomal DNA during growth. Traditionally, only one isolate per farm has been stored in a national collection. However, an infection may have had existed subclinically for a long time in the herd before the first *Salmonella* isolations were obtained. When that occurs, a clear difference in the banding patterns within the herd may be seen. If similar differences were seen between single isolates from different herds or infection sources, it would be extremely difficult to judge the significance of the finding, especially in case of an endemic infection. Analysis of several and successive isolates from each herd gives perspective for the interpretation of differences in banding patterns arising during a single infection.

The ability to compare results from different laboratories at a regional, national and international level is increasingly important with the free movement of goods and people. Olsen *et al* (1997) stated that there clearly is a need for standardization of molecular typing procedures to maximize this ability to compare typing results. Furthermore, Fisher (1999) published a description on how the Enter-net international surveillance network works. For *Salmonella*, serotyping, phage typing of epidemiologically important serotypes, and antibiogram determination have been harmonized in all participating countries (17 European and five non-European) and the results pooled. In addition to Enter-net, Salm-gene (Peters *et al* 2003) was established to evaluate the added value of using molecular subtyping (namely PFGE) for food-related salmonellosis. The national reference laboratories of countries in Europe participated in the project (Austria, Denmark, Finland, Germany, Italy, The Netherlands, Scotland, Spain, England, and Wales). The PFGE laboratory procedures were standardized; to facilitate comparison with other international work, the electrophoresis conditions were identical to those used by PulseNet USA, and the gel images were entered into and analysed in the Salm-gene database. It consisted of PFGE profiles of almost 25 000 *Salmonella* strains with epidemiological data from nine participating countries.

In Finland, *Salmonella* serotypes are subtyped by *Xba*I-PFGE (and *Bln*I-PFGE or plasmid analysis when needed) at the National Public Health Institute (Lukinmaa *et al* 2004) or at the Finnish Food Safety Authority Evira. By 2007, more than 3000 *Salmonella* strains had been genotyped and the various PFGE profiles stored in an electronic library. The situation on the most common serovars causing infection in humans and animals in Finland has recently been reviewed (Siitonen A, Kuronen H, Pelkonen S *in press*). In the 2000s, fewer than 3000 cases per year of salmonellosis in humans were recorded. Less than 20 per cent of these cases were of domestic origin.

Approximately 50 different serovars were annually isolated from the domestic cases of salmonellosis. In the cases of salmonellosis of foreign origin, approximately 100 different serovars were detected each year. The isolated serovars varied from one year to another. However, only ten serovars caused about 75 per cent of the infections. *S*. Typhimurium and *S*. Enteritidis were frequently isolated from infections of both domestic and foreign origin.

Salmonella is rare in Finnish production animals. Less than 1 per cent of the specimens have been positive for *Salmonella*, thereby attaining the aim of the Finnish *Salmonella* control programme (FSCP) mentioned in Sections 2.3 and 2.4. *S*. Infantis and *S*. Typhimurium, especially DT1, are the most common serovars isolated from Finnish production animals (poultry, pigs and cattle). *S*. Infantis used to be the predominant *Salmonella* serovar in broiler chickens. However, since 2001, *S*. Livingstone has been the most commonly isolated serovar. *S*. Typhimurium was only isolated in one batch in 2001. *S*. Enteritidis was not detected in broiler chickens at all. In turkeys, *S*. Typhimurium was the most commonly isolated serovar. Eighteen different serovars were recorded in pig slaughterhouses, and about 30 serovars in cattle herds. The most common serovar in isolates from the 2000s was *S*. Typhimurium.

Sporadical isolations were made from other production animals, pets, wild animals, fur animals, and zoo animals. *S*. Typhimurium was the most commonly detected serovar, followed by *S*. Enteritidis. *S*. Poona was the dominating serovar in isolates from fur animals. *S*. Typhimurium phage types DT40 and U277 were found in small birds and hedgehogs. These phage types also commonly infected humans. However, *S*. Typhimurium DT41, seen in seagulls, was rarely isolated from human cases of salmonellosis. In the 2000s, almost one third of the analysed *Salmonella* strains were of another subspecies than *Salmonella enterica* subsp. *enterica*. These subspecies were seen in reptiles, and were also detected in human cases of salmonellosis.

7. CONCLUSIONS

1. The genotype of the feedstuff-related *S*. Infantis strain was identified. It belonged to the major endemic type pf1, but differed from it by having a plasmid visible as an intensive 60 kb band with *Xba*I-PFGE (plasmid subtype pf1/39). Farms infected with the feedstuff-related genotype pf1/39 or the related genotypes pf1/43, pf1/44, pf1/45 or pf1/46 carrying the same 60 kb plasmid were identified. The feedstuff-related plasmid profile was stable on the infected farms during the follow-up period.

2. *S*. Infantis infection in cattle was highly clonal, as 99 per cent of the isolates had *Xba*I-PFGE profiles clonally related to each other. The major genotype pf1 was predominant both at the starting year of our analysis in 1985 and as the infection seemed to fade out in 2003. There was no trend towards increasing diversity. The feedstuff-related outbreak strain of 1995 did not persist in the cattle population. However, there was a general decline in bovine salmonella infections from 1997 onwards. Testing of several isolates obtained from a herd in outbreak investigations is advisable since minor changes in banding pattern frequently occur during long-lasting infections.

3. *S.* Agona isolates causing a small outbreak on cattle farms in 1994-1995 period were shown to contain closely related genotypes, when characterized by PFGE using *Xba*I, *Bln*I, *Spe*I and *Not*I enzymes. Another possibly genetically related small outbreak occurred in cattle in 1997. Based on our typing results, the large human outbreak in 1999 was not related to the cattle farm outbreaks. Thus molecular typing could show that although the outbreaks were regionally clustered, to our knowledge they were not related in any other way.

4. Two clusters were formed among *S*. Typhimurium DT1 by compilation of the *Xba*I-, *Bln*I- and *Spe*I-PFGE and IS*200*-types and possession of the serovar-specific virulence plasmid. The major cluster contained eight IS*200*-types, including the most common IS*200*-type D and the *Xba*I-PFGE profile 10, but it had no virulence plasmid. The absence of the virulence plasmid can be regarded as typical of this endemic infection. Combination of the PFGE results gave 54 different combination profiles. The source for sporadic human infections is unknown in most cases and genetic typing did not suggest any clear infection source. However, the most common *Xba*I-profile 10 was seen in analysed isolates obtained from hedgehogs and wild birds. These sources might act as important reservoirs, maintaining a minimum baseline level of *S*. Typhimurium DT1 in the environment and consequently, be possible sources for human infections.

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