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Persistence of *Salmonella* Senftenberg in poultry production environments and investigation of its resistance to desiccation

Tina Broennum Pedersen^{1*}, John Elmerdahl Olsen¹ and Magne Bisgaard¹

¹Department of Veterinary Pathobiology, Faculty of Life Sciences, University of Copenhagen, Stigbøjlen 4, DK-1870 Frederiksberg C, Denmark

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*Corresponding author. Department of Veterinary Pathobiology, Faculty of Life Sciences, Copenhagen University, Stigbøjlen 4, DK-1870 Frederiksberg C, Denmark,

Phone: + 45 35 33 34 28, Fax: + 45 35 28 27 57, email: tbp@life.ku.dk

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Persistence of *Salmonella* Senftenberg in poultry production environments and investigation of its resistance to desiccation

Tina Broennum Pedersen^{1*}, John Elmerdahl Olsen¹ and Magne Bisgaard¹

Abstract

Most *Salmonella* serovars, including *Salmonella enterica* subsp. *enterica* serovar Senftenberg (*S.* Senftenberg), are tolerant to desiccation and able to colonise and persist in feed mills. In addition, they may survive cleaning and disinfection procedures used on poultry farms. This study was conducted to investigate the survival of *S.* Senftenberg in broiler parent stock (PS) farms and broiler farms. The isolates from one of the parent stock farms investigated only differed by a single band in fluorescent amplified fragment length polymorphism analysis (AFLP) and had identical pulsed field gel electrophoresis (PFGE) profiles, indicating that a *S.* Senftenberg clone had persisted for more than two years, despite cleaning, disinfection, desiccation and depopulation, and was subsequently able to infect *Salmonella* free layers. Isolates from the same house on a different broiler PS farm were found to be identical by AFLP and PFGE although the farm tested negative for *Salmonella* 55 times over a period of 18 months between the two positive samplings. An assay was developed to investigate the survival of 34 *S.* Senftenberg isolates during desiccation at approximately 38% relative humidity. On average, the viability of *S.* Senftenberg isolates decreased by 1000 fold over 35 days. The persistent clones were not any more resistant to desiccation than the other isolates investigated. However, *S.* Senftenberg was more resistant to desiccation than an isolate of *Pantoea agglomerans* commonly found on poultry feed processing lines. This study demonstrates the risk of persistence of feed-associated serovars such as *S.* Senftenberg.

Introduction

Salmonella enterica subsp. *enterica* serovar Senftenberg (*S.* Senftenberg) is frequently isolated from feed raw materials, the processing line and environmental samples from feed factories and from poultry farms (Bailey *et al.*, 2001; Nesse *et al.*, 2003; Anonymous, 2006a; Broennum *et al.*, 2007b). In addition, *S.* Senftenberg has been associated with several food-borne outbreaks of salmonellosis in humans (Rushdy *et al.*, 1998; Mohle-Boetani *et al.*, 2001).

The routes of introduction of *Salmonella* into a broiler flock include vertical transmission from *Salmonella* infected parent flocks, persistence in the environment and contaminated feed (Bisgaard, 1998). In 1994 Denmark implemented EU directive 92/117/EEC ("the Zoonosis Directive"), under which all breeding stock are tested for *S.* Enteritidis and *S.* Typhimurium, with elimination of positive flocks. By December 1996, the Danish *Salmonella* Control Program was extended to include all *Salmonella* serovars, and eggs from a flock can only be used for production of heated egg products if any *Salmonella* type is detected. The Danish Poultry Council, in cooperation with feed mills, has run a voluntary HACCP-based hygiene programme that includes heat treatment since 1989 (Bisgaard, 1992) and heat treatment of animal feed became compulsory in 1993. These initiatives resulted in a significant decrease in the prevalence of *Salmonella* positive broiler flocks and table egg flocks, with current annual prevalences of approximately 2% and 1%, respectively (Anonymous, 2006a). Surprisingly, the prevalence of isolation exotic serovars has not decreased, indicating that feed and/or the production environment remain significant sources.

S. Senftenberg is able to colonize and persist in feed mills and poultry houses (Wray *et al.*, 1990; Anonymous, 2006a; Anonymous, 2006b; Löfström *et al.*, 2006) and it has been

suggested that it is more resistant to stresses such as low pH, heating, desiccation and irradiation than other serovars (Liu *et al.*, 1969; Mackey and Derrick, 1982). However, understanding of the reasons for its increased resistance and unambiguous demonstration of its persistence, as demonstrated by molecular typing methods, have been lacking. This study was conducted to investigate how long *S. Senftenberg* might persist in cleaned and disinfected poultry houses and be able to infect new *Salmonella*-free stock, and to examine whether persistent *S. Senftenberg* isolates survived desiccation better than other *S. Senftenberg* isolates.

Materials and methods

Bacterial strains. A total of 34 *S. Senftenberg* strains were investigated for persistence using amplified fragment length polymorphism (AFLP) and identical pulsed field gel electrophoresis (PFGE) analysis. The 34 strains included two isolates from 2002 and a single isolate from 2004 from the same farm (Farm 3), as well as two isolates from Farm 4 from January 2000 and July 2001 (Table 1). After slaughter of the *S. Senftenberg* positive broiler parent stock (PS) on Farm 3 in February 2003, the farm was cleaned, disinfected and remained free of poultry for 10 months until December 2003. Subsequently, *Salmonella* free stock for table egg production were introduced. Sampling of the flocks was carried out according to national legislation (Anonymous, 2004). In December 2004, the table-egg producing flock tested positive for *S. Senftenberg*, 22 months after the farm had been properly cleaned and disinfected and after six consecutive samples were negative for *Salmonella*. Two pairs of sock samples, obtained by wearing the socks over the boots during a walk through the entire poultry house (Skov *et al.*, 1999), were collected from each house and 60 eggs were tested each time (Anonymous, 2004). Another broiler parent flock (Farm 4) tested positive for *S. Senftenberg* approximately three months before slaughter after having tested negative on a weekly basis for 27 weeks. Subsequently, the flock tested negative 10 times until processing. After cleaning, disinfection and dry-out, a subsequent flock tested negative for the whole production period (40 times). A subsequent flock tested negative for five weeks, after which the house again tested positive for *S. Senftenberg*. However, 35 subsequent samplings all tested negative for *Salmonella*. Full cleaning and disinfection had been carried out in between the flocks.

Twenty-nine other *S. Senftenberg* isolates, from a variety of epidemiological situations and from different time points (Table 1) were included to investigate diversity. All the *S. Senftenberg* isolates had been serotyped using the Kauffmann White Scheme (Popoff & Le Minor, 1997). Single isolates of *Pantoea agglomerans* and *Enterobacter purinus* were used in the desiccation assay as reference strains. These isolates had been found persisting on a poultry feed processing line (Broennum *et al.*, unpublished data)

Desiccation assay. Pure cultures of all strains were inoculated into 10 ml sterile Luria Bertani (LB) broth (Sambrook *et al.*, 1989) and incubated at 37°C for 24 h. Cells from 1 ml of this overnight (ON) culture were harvested by spinning at 15,000 rpm for 5 min at 4°C. The pellets were suspended in 1 ml MilliQ-water to avoid salt stress during desiccation. Aliquots of 50 µl were added to wells in microtitre trays (Nunc A/S, Roskilde, Denmark). The trays were dried at 37°C in an incubation chamber at approximately 70% relative humidity (RH) for 24 h, after which they were transferred to a desiccator maintained at 38-40% RH and at 37°C for the remainder of the study (34 days). Culturable cell counts were performed on samples subjected to desiccation for 0, 1, 2, 5, 9, 15, 20 and 35 days. The desiccated cells were rehydrated by adding 100 µl physiological saline, incubating for 10 min and then mixing by pipetting before plating. Colony forming units (CFU) per ml were calculated by inoculating 10 µl samples from tenfold dilution series onto LB agar plates (Sambrook *et al.*, 1989).

AFLP analysis. Strains were grown overnight in LB broth (Sambrook *et al.*, 1989) at 37°C and DNA was extracted using the EASY-DNA kit (Invitrogen, Carlsbad, CA, USA). AFLP analysis was performed as described by Torpdahl and Ahrens (2004) using *BspDI* and 6-carboxyfluorescein (FAM) labelled *BglIII* primers. GeneScan 3.1 fragment analysis software

(Applied Biosystems, Nærum, Denmark) was used for fragment size determination and pattern analysis. Only AFLP profiles comprising fragments in the size range of 50–500 bp were considered for numerical analysis. This analysis was performed on data files comprising both bacterial AFLP profiles and internal size standards (ABI GeneScan™-500 ROX, Applied Biosystems, Nærum, Denmark) using the program Gelcompar II version 3.5 (Applied Maths, St. Martens-Latem, Belgium). Normalised AFLP fingerprints were compared using the Dice similarity coefficient and clustering analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA). AFLP fingerprints consisting of approximately 65 to 89 fragments per isolate were compared. The arbitrary similarity (S_D) cut off level for individual clonal types was set at 98%.

PFGE analysis. PFGE was performed as described by Christensen *et al.* (1994), using *Xba*I to digest chromosomal DNA. DNA fragments were separated in 1% SeaKem GTG agarose (Camprex Bio Science, Copenhagen, Denmark) using the CHEF DRIII system set at 14°C and 5.6 V/cm with pulses ramping from 1 to 35 s over 20 h . The DNA fragments were stained with ethidium bromide and photographed with ultraviolet transillumination using a Geldoc 2000 system (BioRad, Herlev, Denmark) and Quantity One 4.0.3 gel (BioRad, Herlev, Denmark) documentation software. The images of the gels were saved in TIFF format and imported into the program Gelcompar II version 3.5 (Applied Maths BVBA). Cluster analysis was performed using the Dice correlation for band matching with a 1% position tolerance and UPGMA was used to generate a dendrogram describing the relationships between the *S.* Senftenberg isolates.

Results

Analysis of persistence by molecular typing. A total number of 34 *S. Senftenberg* isolates were analysed by AFLP. Cluster analysis generated a dendrogram with all similarities above 84% (Figure 1). The AFLP profiles consisted of 65 to 89 fragments between 50 and 500 bp. Twenty-one isolates made up one large cluster (A) based on an arbitrary threshold point of 98.7% similarity, and this cluster included isolates obtained from both the farms suspected to have had persistent contamination (Farm 3 and 4), isolates from Farm 1, and isolates from feed raw materials, imported turkey and chicken meat, and imported curry. This cluster could be divided into subclusters A1, A2, A3 and A4. All three isolates from Farm 3 belonged to subcluster A1. Two isolates from 2002 and 2004 (21 and 22, respectively) were identical, while a second isolate from 2002 (24) differed from these two by a single band. Two isolates from imported curry and single isolates from imported chicken and turkey meat shared the same AFLP profile and made up subcluster A2. Nine isolates from a broiler farm (Farm 1) and a broiler breeder farm (Farm 4) made up subcluster A3. Isolates from Farm 1 were obtained between February 2004 and May 2005, while isolates from the breeder farm were obtained between January 2000 and July 2001. Subcluster A4 included isolates from soy, process line samples from a feed mill, and one isolate from Farm 1. These isolates differed by four bands.

Cluster B had 98% similarity to cluster A and included isolates from imported turkey meat and exported swine meat. Two isolates from poultry feed processing line samples, which grouped together with one isolate from Farm 2, formed cluster C (Figure 1), although there were 16 band differences between these isolates. Isolates in cluster D had least similarity to the other isolates. This cluster also included a feed mill process line isolate and an isolate from Farm 5. However, there were eight band differences between these isolates.

Cluster E included two highly related isolates from imported chicken meat. In addition to these five clusters, there were single isolates with distinct profiles.

The 34 *S. Senftenberg* isolates were also subjected to PFGE analysis. A subset of 25 isolates is shown in Figure 2. All isolates from Farm 1 had identical profiles. Likewise, isolates 28 and 33 had identical profiles, as well as isolates 18 and 19 (not shown). PFGE separated the isolates into 15 profiles with less than 90% similarity (Figure 2). Six clusters (I-VI) had greater than 90% similarity. Isolates in cluster I had identical profiles and included all the isolates obtained from Farm 3. All isolates from Farm 4 (cluster II) were identical, as were those from Farm 1 (cluster III). Isolates from Farm 4 were similar to isolate 34, which was obtained from a sample of soy. Isolates from imported turkey meat in cluster IV were also similar, as were isolates from imported turkey and pig meat in cluster V, and isolates from imported curry and process line samples in cluster VI.

Resistance to desiccation. The mean concentration of viable cells after overnight culture of all the *S. Senftenberg* isolates was $9.64 \log_{10}$ CFU/ml. After the cells were washed and dispensed into the wells there was a mean loss of viability of approximately 13%, leaving a mean concentration of $9.57 \log_{10}$ CFU/ml for the desiccation trial. Over the first 24 hours the concentration of viable cells decreased by 79% to approximately $8.87 \log_{10}$ CFU/ml (Figure 3). After 48 hours there was a further decrease in concentration of only 6%. Over the first 20 days there was an 83% decrease in the concentration of viable cells and a further decrease of 36% between days 20 and 35. Over 35 days of desiccation at 40% RH, there was a 99.8% reduction in the concentration of viable cells, to a final concentration of $6.12 \log_{10}$ CFU/ml.

The variation between isolates increased with duration of desiccation (Figure 3), and the concentration of the different strains at day 35 ranged from 4.22 to $7.77 \log_{10}$ CFU/ml (Figure 3). The loss in viability of the three isolates from Farm 3 was less than the mean of all

the *S. Senftenberg* isolates (data not shown). However, strains isolated from turkey meat (numbers 11 and 14), a feed mill processing line sample (number 27), and a sample of soy (number 32) showed the greatest resistance to desiccation. There was no significant difference in resistance to desiccation between the isolates from the different sources (farms, meat, spices and feed mills). The resistance of the *Enterobacter purinus* isolate was similar to that of the *S. Senftenberg* isolates, while the *Pantoea agglomerans* isolate lost viability rapidly and the concentration of viable cells was only 1.3 log₁₀ CFU/ml after 35 days of desiccation (Figure 3).

Discussion

We investigated the persistence of *S. Senftenberg* in poultry environments. The three isolates obtained from Farm 3 over a period of 30 months were identical by PFGE analysis and by AFLP analysis the isolates obtained in July 2002 and December 2004 were identical while a single isolate obtained in August 2002 differed from them by only one DNA fragment. These data suggest that *S. Senftenberg* can persist for very long periods of time. Over this 30 month period, the environment of the poultry house was exposed to detergents, disinfectants and subsequent desiccation. This observation extends those of Davies and Wray (1996), who were able to isolate *Salmonella* Enteritidis PT4 from floor sweepings and rehydrated wall fabric junctions in an empty poultry house 22 weeks after cleaning and disinfection. However, their characterization relied on serotyping and phage typing, whereas we used two different genotyping methods and serotyping to demonstrate clonality. The ability of *S. Senftenberg* to persist in poultry production facilities and infect subsequent stock was also demonstrated by observations on both Farms 1 and 4 in our study.

Despite the demonstration of persistence of *S. Senftenberg* on Farms 3 and 4, salmonella were not isolated from repeated weekly samples over extended periods. *Salmonella* were not isolated from the broiler parent flock on Farm 3 during rearing or the first three weeks on the production farm prior to the first positive sample from Houses 1 and 6 in July 2002. The following week, a positive sample was also obtained from House 2, while samples from the other houses were negative. After two weeks of negative samples, a sample from House 1 was again positive, then samples for an additional 12 weeks were negative, followed by a positive sample and a further 13 negative samples. Samples from House 6 were negative every week until the eleventh week after the first positive sample, then this positive sample was followed by 18 negative samples. A total of 55 samples tested negative for

Salmonella between these two samplings. Similar observations of samples intermittently positive for *S. Enteritidis* and/or *S. Typhimurium* have been reported previously (Gradel & Rattenborg, 2003; Anonymous, 2006b). The intermittently positive samplings might be explained by either a lack of sensitivity of the sampling and test method, combined with a very low infection pressure (Skov *et al.*, 1999). However, as doses as low as 5 cells (Milner & Schaffer, 1952) or 10^2 cfu (Van Immerseel *et al.* 2004) have shown to be able to colonize chickens, the possibility of persistence in a location to which the chickens are infrequently or indirectly exposed should be considered. Age differences in susceptibility to infection (Beal *et al.*, 2004; Van Immerseel *et al.* 2004) might also partially explain the intermittently positive samplings.

Serovars such as *S. Senftenberg* and *S. Infantis* appear to persist in poultry houses (Gradel & Rattenborg, 2003; Anonymous, 2006b). These persistent serovars have also been found in raw materials for poultry feed production (Bisgaard, 1998; Anonymous, 2004; Broennum *et al.* unpublished data). Although *Salmonella* were not isolated from any finished poultry feed in Denmark over the period in which these isolates were obtained from the farms (Anonymous, 2004; Broennum *et al.* unpublished data), epidemiological investigations indicate that feed still represents a risk to the poultry industry (Davies *et al.*, 1997; Bisgaard, 1998; Jensen & Rosales, 2002; Broennum *et al.* unpublished data). PFGE analysis of isolates from Farms 3 and 4 suggested that they were related to isolates from feed raw materials. Since the isolates from soy were obtained in July 2002 (Feed Mill A) and November 2003 (Feed Mill C) and the isolates from Farm 4 were from January 2000 and July 2001, there does not appear to be any direct link. However, as Feed Mills A, B and C sometimes share bulk imported raw feed materials and as *S. Senftenberg* may persist in bulk feed tanks and other parts of the unclean area of the a feed mill (Nesse *et al.*, 2003; Anonymous, 2006b; Löfström

et al., 2006), the poultry feed mill might have been contaminated with *S. Senftenberg* from soy products imported prior to 2000. Soy seems to pose a particular risk for *Salmonella* contamination (Anonymous, 2006a; Broennum *et al.*, unpublished data).

Finished poultry feed and feed raw materials are normally stored at 12-14 % RH to prevent microbial growth. The water activity (a_w) and organic matter in the environment or in feed influence the survival of *Salmonella* during desiccation (Mattick *et al.*, 2000; Mattick *et al.*, 2001). Other feed ingredients, such as fat, also influence bacterial survival (Greenwood & Hopper, 1983). Duncan and Adams (1972) suggested that in feeds with similar moisture contents, different ingredients may affect a_w values, providing different environments for bacterial survival and growth. As the desiccation assay used in this study was based upon dehydration of the cells after washing in MilliQ-water, enhanced survival due to lipids, proteins and cross-protection by adaptation to salt were excluded. Over 35 days of desiccation at 40% RH, we observed a mean 99.8% reduction in the concentration of viable cells of the 34 *S. Senftenberg* isolates. Ha *et al.* (1998) observed a 66% decrease in the concentration of *Salmonella* after 56 days in poultry mash containing soybean meal and a 71% decrease in mash containing meat and bone meal (a_w approx. 0.63%). We found that all the *S. Senftenberg* isolates survived better than a *Pantoea agglomerans* isolate. However, *S. Senftenberg* has not previously been found to persist any longer in poultry feed than *S. Enteritidis*, *S. Typhimurium* or *S. Mbandaka* (Davies & Wray, 1996). The *Pantoea agglomerans* isolate used in our study has previously been shown to persist over a period of 14 days in the cooler in a processing line producing poultry feed, where the RH of the feed was below 20% (Broennum *et al.*, unpublished data).

Our desiccation study was performed at 40% RH and 37°C. Thirty-one *S. Infantis* isolates desiccated under similar conditions had comparable losses of viability and the losses in viability at 37°C were similar to those seen at 20°C over 35 days (Broennum *et al.*,

unpublished data). Therefore, although our study of resistance of *S. Senftenberg* to desiccation was performed at 37°C, it may still be indicative of survival in poultry houses, in which ambient temperatures range from 34°C to 18°C between the introduction of day old chicks and the time of slaughter.

Previous epidemiological studies on the persistence of *Salmonella* in poultry environments have used serotyping and plasmid profiling (Baggesen *et al.*, 1992; Brown *et al.*, 1992). In our investigation, a combination of fluorescently labelled AFLP and PFGE analysis was used to ensure reliability, as the discriminatory power of typing methods differs between *Salmonella* serotypes (Liebana *et al.*, 2001a). PFGE of fragments generated by *Xba*I digestion of genomic DNA has been recognised as a sensitive method for molecular fingerprinting of several *Salmonella* serotypes (Lyytikainen *et al.*, 2000; Valdezate *et al.*, 2000; Liebana *et al.*, 2001b).

AFLP has previously been used for separation of epidemiologically unrelated isolates within serovars of *Salmonella enterica* with varying results (Lindstedt *et al.*, 2000; Torpdahl & Ahrens, 2004). A threshold of greater than 90% similarity has been used previously to define genetically similar isolates of *S. Typhimurium* DT 126 in fluorescent AFLP studies (Ross & Heuzenroeder, 2005). However, use of a threshold of 90% similarity for AFLP analysis of *S. Senftenberg*, did not generate the same clusters as obtained using PFGE, suggesting that the threshold similarity for AFLP analysis must be determined empirically for each organism to enable meaningful biological and epidemiological conclusions to be drawn.

In conclusion, *S. Senftenberg* was demonstrated to persist in several poultry production environments for periods of up to 30 months. In addition, we demonstrated that *S. Senftenberg* was relatively resistant to 35 days of desiccation at 40% RH. Finally, our study highlights the problems associated with reisolation of *Salmonella* on farms and underlines the

risk of persistence related to the introduction of feed-associated *Salmonella* serovars such as *S. Senftenberg*

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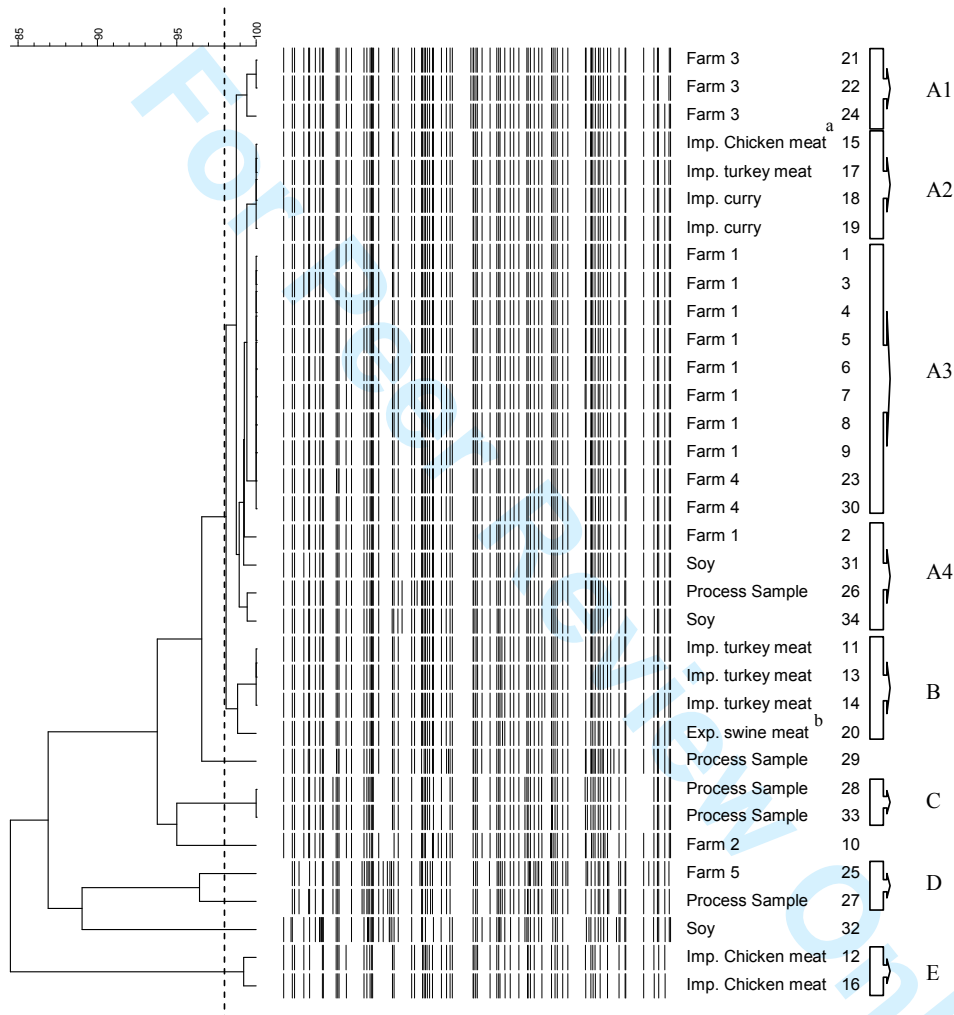
Table 1. *S. Senftenberg* isolates analysed by AFLP and PFGE and subjected to desiccation

Isolate No.	Isolation date	Origin	Remarks
1	February 2004	Farm 1	Isolate from sock sample, broiler production
2	February 2004	Farm 1	Isolate from sock sample, broiler production
3	March 2004	Farm 1	Isolate from sock sample, broiler production
4	March 2004	Farm 1	Isolate from sock sample, broiler production
5	March 2004	Farm 1	Isolate from sock sample, broiler production
6	November 2004	Farm 1	Isolate from sock sample, broiler production
7	November 2004	Farm 1	Isolate from sock sample, broiler production
8	May 2005	Farm 1	Isolate from sock sample, broiler production
9	May 2005	Farm 1	Isolate from sock sample, broiler production
10	July 2004	Farm 2	Isolate from sock sample, broiler production
11	May 2003	Imported meat	Turkey meat, import control
12	October 2003	Imported meat	Chicken meat, import control
13	January 2004	Imported meat	Turkey meat, import control
14	August 2004	Imported meat	Turkey meat, import control
15	December 2004	Imported meat	Chicken meat, import control
16	February 2005	Imported meat	Chicken meat, import control
17	March 2005	Imported meat	Turkey meat, import control
18	March 2003	Imported spices	Curry
19	March 2003	Imported spices	Curry
20	April 2003	Meat	Swine meat, export control
21	July 2002	Farm 3	Isolate from sock sample, broiler parent production
22	December 2004	Farm 3	Isolate from sock sample, table egg production
23	January 2000	Farm 4	Isolate from sock sample, broiler parent production
24	August 2002	Farm 3	Isolate from sock sample, broiler parent production
25	January 2002	Farm 5	Isolate from sock sample, broiler parent production
26	January 2002	Feed Mill A	Process sample
27	December 2003	Feed Mill B	Process sample
28	January 2004	Feed Mill C	Process sample
29	January 2004	Feed Mill C	Process sample
30	July 2001	Farm 4	Isolate from sock sample , broiler parent production
31	July 2002	Feed Mill A	Soy, raw feed material sample
32	July 2002	Feed Mill A	Soy, raw feed material sample
33	November 2003	Feed Mill C	Process sample
34	November 2003	Feed Mill C	Soy, raw feed material sample

Figure 1. *AFLP fingerprints of 34 S. Senftenberg strains. Dendrogram calculated using GelCompare II. The broken line indicates the division into clusters based upon 98% similarity with the Dice coefficient*

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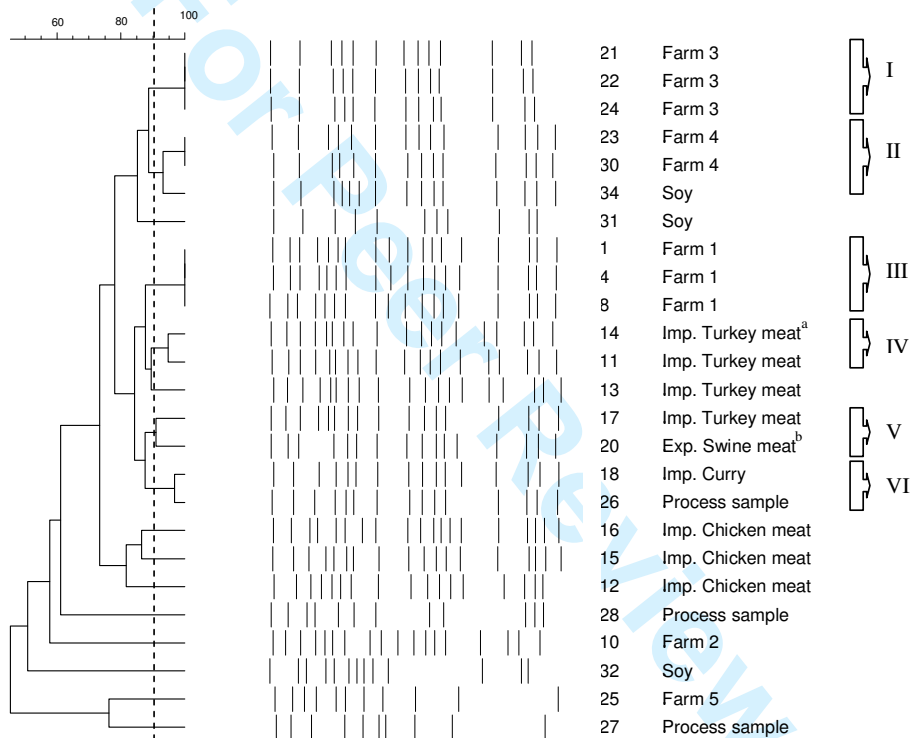


^a Imp., imported; ^b Exp., exported

Figure 2. *Dendrogram showing PFGE fingerprints from 25 isolates of Salmonella Senftenberg representing the total diversity of isolates included in this study. Using the unweighted pair group method with arithmetic averages (UPGMA), cluster analysis was performed with the Dice correlation method. The position tolerance was set to 1% and optimisation was 1%. The broken line indicates the division into clusters made at 90% similarity with the Dice coefficient*

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^a Imp., imported; ^b Exp., exported

Figure 3. Mean concentrations of 34 isolates of *S. Senftenberg* (filled square) during desiccation at 40% relative humidity. The error bars show the range of concentrations. Concentrations of one isolate of *Enterobacter pyrinus* (open triangle) and one of *Pantoea agglomerans* (filled diamond) are also shown

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