

Brief Original Article

## Clonal persistence of *Salmonella enterica* serovars Montevideo, Tennessee, and Infantis in feed factories

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### Abstract

**Introduction:** Novel molecular techniques applied in biotechnology research have provided sound evidence on clonal persistence of distinct serovars of *Salmonella* in feed factory environments, over long periods of time (months, even years), which can be responsible for repeated in-house contamination of final products. In this study, we examined the possibility of clonal persistence of isolates of three *Salmonella* serovars that have been repeatedly identified in animal feed samples from three feed factories throughout a two-year period.

**Methodology:** The isolates *Salmonella enterica* serovars Tennessee (n = 7), Montevideo (n = 8), and Infantis (n = 4) were tested for genetic diversity using pulsed-field gel electrophoresis (PFGE) and multicellular behavior patterns by applying the Congo red agar test.

**Results:** *SpeI* and *XbaI* macro-restriction profiles indicated that isolates *S. Montevideo* and *S. Infantis* were identical, whereas isolates of *S. Tennessee* demonstrated greater genetic diversity, although the genetic differences did not exceed 10%. All *Salmonella* serovars demonstrated the ability to produce predominant matrix compounds essential for biofilm formation, curli fimbriae and cellulose.

**Conclusions:** The identification of identical clones of *S. Montevideo* and *S. Infantis*, as well as the minor genetic diversity of *S. Tennessee*, which have been repeatedly isolated from animal feed in three production plants throughout a two-year period, indirectly suggests the possibility of their persistence in feed factory environments. Their ability to express the key biofilm matrix components further supports this hypothesis.

**Key words:** feed; PFGE; *Salmonella* spp.; rdar morphotypes.

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### Introduction

Animal feed is a gateway for microbes to enter the food chain [1]; it is frequently contaminated with foodborne bacterial pathogens [2]. Bacteria of the genus *Salmonella* are of particular importance, since their presence in animal feed is associated with a high risk of bacterial colonization or infection in animals and, consequently, infection in humans consuming products of animal origin [1,2]. The link between *Salmonella* in animal feed and human salmonellosis has been well established for many years [2,3].

Ingredients of plant and animal origins are a common route for introduction of *Salmonella* spp. into animal feed. There is limited knowledge about the fact that feed ingredients are a major risk factor for the contamination of production equipment in feed factories, which makes them a permanent source of in-house contamination [4-6]. The development of novel molecular techniques (pulsed-field gel electrophoresis [PFGE] and plasmid profile typing) offered evidence that clones of diverse *Salmonella* serovars can persist in

feed factory environments for months or even years [4,6-9]. The mechanisms activated by *Salmonella* spp. enabling its survival outside the host rely on multicellular behavior patterns resulting from the expression of specific genes and synthesis of products encoded by these genes. The production of curli fimbriae and cellulose is considered crucial for the survival of *Salmonella* in the environment [10,11]. *In vitro* testing is commonly performed using isolate cultivation on Congo red agar and identification of colony morphotypes. Rdar morphotype (red, dry, and rough) is characteristic for curli- and cellulose-producing isolates, and is a synonym for bacterial communities that are able to form an extensive biofilm on abiotic surfaces [12].

In this study, we examined the possibility of clonal persistence of isolates of three *Salmonella* serovars that have been repeatedly identified in animal feed samples from three feed factories throughout a two-year period, using PFGE and the Congo red agar test.

## Methodology

### Bacterial isolates, collection, and identification

*Salmonella* spp. isolates were collected during two years of research (2012–2013) from samples of feed that originated from three production facilities (A, B, C). Food sampling was performed once a month. In cases where two or more *Salmonella* isolates of the same serological group were isolated from two or more samples taken on the same day, only one isolate was included in the research. Further research was conducted on three *Salmonella enterica* subsp. *enterica* serovars, Tennessee (n = 7), Montevideo (n = 8), and Infantis (n = 4).

Isolation and biochemical identification of *Salmonella* spp. was performed pursuant to the International Organization for Standardization (ISO) protocol 6579:2002 [13]. Serotyping of isolates was performed in the National Reference Laboratory for *Salmonella*, *Shigella*, *Vibrio cholerae*, *Yersinia enterocolitica*, Institute of Public Health of Serbia Dr. Milan Jovanovic Batut, Belgrade. Until testing, the strains were stored in tryptone soya broth (TSB) (CM0129, Oxoid, Basingstoke, UK) with 15% glycerol at -80°C. Overnight cultures cultivated on xylose lysine deoxycholate agar (XLD), (Biokar Diagnostics, Beauvais Cedex, France) at 37°C were used for the examination.

The types and total number of analyzed feed samples as well as the numbers and serovars of *Salmonella* spp. isolates originating from feed factories A, B, and C are displayed in Table 1.

### PFGE

The preparation of samples was performed as previously described [14]. The DNA restriction was done with *Xba*I and *Spe*I enzymes (Thermo Fischer Scientific, Vilnius, Lithuania) at 37°C for 3 hours. PFGE was performed with a 2015 Pulsafor unit (LKB Instruments, Broma, Sweden) equipped with a hexagonal electrode array for 16 hours at 300 V at 9°C. The gels were stained with ethidium bromide and photographed under UV illumination. A dendrogram was derived from the Ward linkage of correlation coefficients between PFGE patterns of different genotypes using SPSS cluster analysis software (SPSS; Chicago, USA). *S. Braenderup* H9812 [MIS-00418] (ATCC, USA) was used as a molecular size marker.

### Congo red agar test

The Congo red agar was prepared from Luria Bertani broth (LB) without salt: Bacto yeast extract (5 g/L), Bacto tryptone (10 g/L) (Becton, Dickinson and Company, Sparks, USA), supplemented with Congo red (40 mg/L) (MP Biomedicals, Illkirch, France) and

**Table 1.** Type and number of animal feed samples originating from feed factories A, B, and C; *Salmonella* serovars and number of isolates

Type and number of feed	Feed factory A		Feed factory B		Feed factory C	
	(n)	No. of isolates and serotypes	(n)	No. of isolates and serotypes	(n)	No. of isolates and serotypes
Cattle feed mix	16	<i>S. Tennessee</i> (3) <i>S. Agona</i> (1)	-	-	23	<i>S. Jerusalem</i> (1)
Pig feed mix	19	<i>S. Tennessee</i> (1) <i>S. Senftenberg</i> (1) <i>S. Enteritidis</i> (1) <i>S. Agona</i> (1) <i>S. Typhimurium</i> (1)	17	<i>S. Montevideo</i> (1)	18	<i>S. Infantis</i> (1) <i>S. Dahra</i> (1) <i>S. Typhimurium</i> (1)
Poultry feed mix	24	<i>S. Tennessee</i> (2) <i>S. Senftenberg</i> (1) <i>S. enterica</i> subsp. <i>enterica</i> (1,3,19:i:-) (1) <i>S. Mbandaka</i> (1)	94	<i>S. Montevideo</i> (7) <i>S. Senftenberg</i> (1) <i>S. Agona</i> (1) <i>S. infantis</i> (1)	25	<i>S. Infantis</i> (2) <i>S. Senftenberg</i> (1)
Soybean meal	5	<i>S. Tennessee</i> (1)	-	-	6	<i>S. Infantis</i> (1)
Sunflower meal	3	-	-	-	5	-
Soybean grits	1	-	-	-	-	-
Maize	4	-	-	-	6	-
Sorghum	1	-	-	-	-	-
Chalk, salt, phosphates	5	-	-	-	-	-
Yeast	5	-	-	-	-	-
Maize silage	-	-	-	-	3	-
Feed mixes for rabbits and pigeons	10	-	-	-	-	-
<b>TOTAL:</b>	<b>93</b>	<b><i>Salmonella</i> spp. = 15 <i>S. Tennessee</i> = 7</b>	<b>113</b>	<b><i>Salmonella</i> spp. = 11 <i>S. Montevideo</i> = 8</b>	<b>86</b>	<b><i>Salmonella</i> spp. = 8 <i>S. Infantis</i> = 4</b>

Coomassie brilliant blue G-250 (20 mg/L) (Sigma-Aldrich, Darmstadt, Germany) ( [15]. All isolates of *Salmonella* serovars Tennessee, Montevideo, and Infantis were inoculated using the spot inoculation technique (single point), without additional spreading. Plates were incubated over 5 days at 20°C. The experiment was done only once in duplicate. The results were interpreted on the basis of characteristic morphotype: rdar (red, dry, and rough; expresses curli and cellulose), pdar (pink, dry, and rough; expresses cellulose), bdar (brown, dry, and rough; expresses curli), and saw (smooth and white; neither curli nor cellulose) [12,16].

**Results**

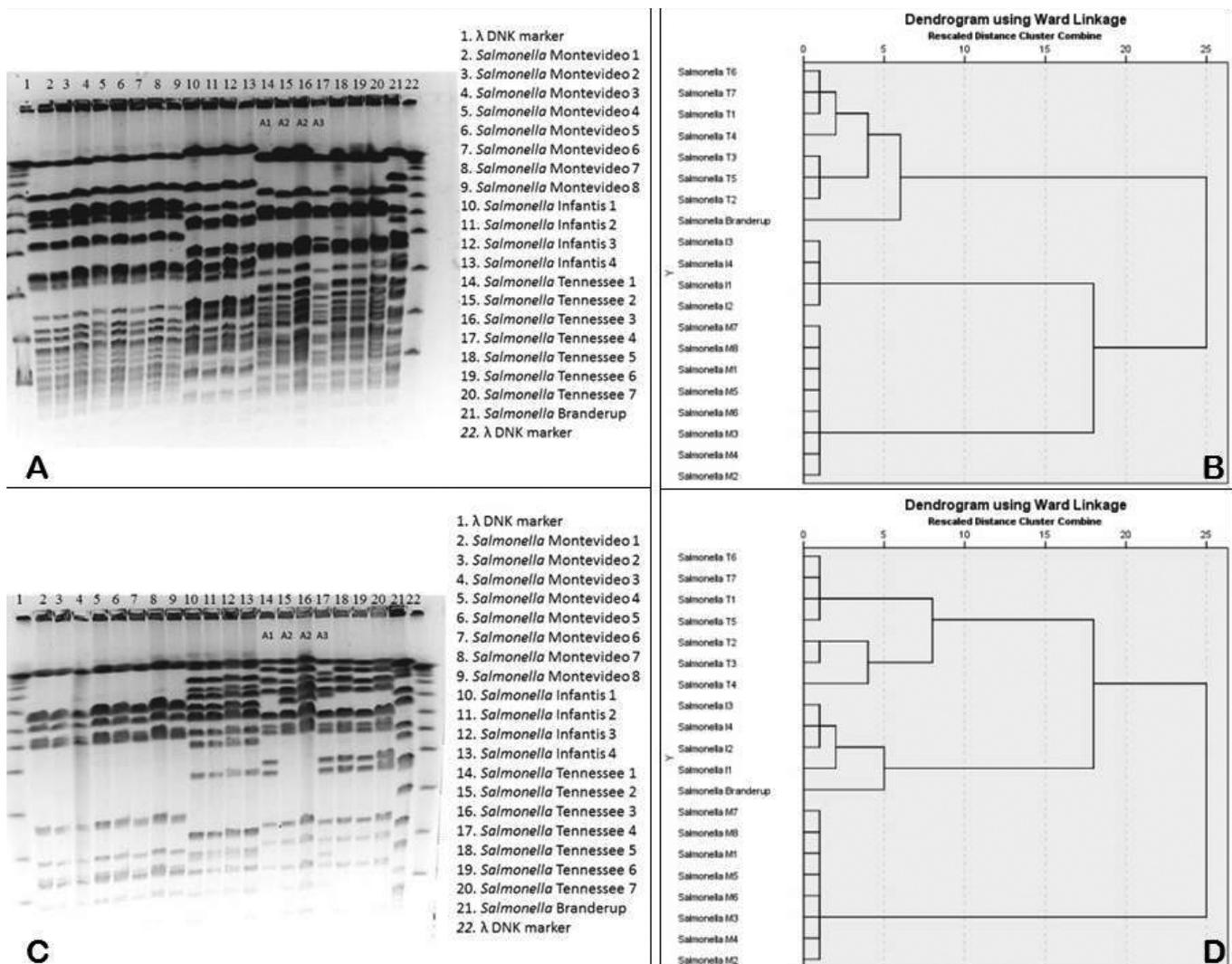
*PFGE*

According to *SpeI* (Figure 1A, B) and *XbaI* macro-restriction profiles (Figure 1C, D), it was established that the *S. Montevideo* isolates were identical, as were the *S. Infantis* isolates, whereas *S. Tennessee* isolates demonstrated the highest genetic diversity, although the difference rate did not exceed 10%.

*Congo red agar test*

*S. Tennessee*, *S. Montevideo*, and *S. Infantis* expressed rdar colonial morphotype, distinctive for curli- and cellulose-producing *Salmonella* strains [12,16].

**Figure 1.** Pulsed-field gel electrophoresis (PFGE) macro-restriction fragment patterns of *S. Tennessee*, *S. Montevideo*, and *S. Infantis*. **A:** Digested with *SpeI* enzyme; **B:** dendrogram derived from the Ward linkage coefficient of correlation between the obtained *SpeI* PFGE macro-restriction profiles; **C:** digested with *XbaI* enzyme; **D:** dendrogram derived from the Ward linkage coefficient of correlation between the obtained PFGE *XbaI* macro-restriction profiles.



## Discussion

Animal feed comprises ingredients of animal and plant origin; thus, sources of contamination with *Salmonella* species are manifold. Feed factory environments represent one of the potential, yet rarely investigated, sources of final product contamination, in spite of the well-known fact that foodborne pathogens such as *Salmonella* spp. can enter the final products by contact with contaminated processing equipment.

Repeated isolation and higher prevalence of *S. Tennessee*, *S. Montevideo*, and *S. Infantis* in production plants A, B, and C, may result from their persistence in feed factory environments. We investigated their genetic diversity and multicellular behavior patterns. The PFGE analysis showed that *S. Infantis* and *S. Montevideo* are indistinguishable and hence present identical clones. The macro-restriction patterns with *Xba*I enzyme in *S. Tennessee* isolates from feed factory A suggest clonal persistence; however, slight genetic differences were noticed. The most prevalent pulsotype in *S. Tennessee* was A1, while in the A2 group, an additional large fragment was found. In the A3 group, one large fragment was missing and one large fragment was detected, as in the A2 group (Figure 1C). Macro-restriction patterns with *Spe*I enzyme suggested that the most prevalent pulsotype in *S. Tennessee* was A1. In two isolates from the A2 group, one additional large fragment was noticed. In the A3 group, one large fragment of a different size was detected in isolate number 17 (Figure 1A). According to Tenover *et al.* [17], isolates are indistinguishable if their restriction patterns have the same numbers of bands and the corresponding bands are the same apparent size, while isolates are closely related if two- to three-band differences are caused by a single genetic event. The statistical analysis has shown that the genetic differences were less than 10%, suggesting the clonal nature of *S. Tennessee*. Nesse *et al.* [4] found only two distinct PFGE types in the four most prevalent *Salmonella* serovars, Senftenberg, Agona, Montevideo, and Kentucky, which were isolated from fish feed factories.

Even though the hygiene routines in animal feed production plants imply the maintenance of dry production environments, temperature changes and consequent condensation provide enough humidity to promote the development of bacterial biofilms, including *Salmonella* [9]. The formation of *Salmonella* species biofilm on abiotic surfaces is part of their life cycle [11,18]. All isolates of *S. Montevideo*, *S. Tennessee*, and *S. Infantis* examined in this research demonstrated the ability to produce predominant matrix

compounds, curli fimbriae (alternatively called tafi or thin aggregative fimbriae in *Salmonella*) and cellulose, which are of key importance in biofilm formation [8,18]. Curli fimbriae are adhesive structures that play a crucial role in the initial stages of adhesion to biotic and abiotic surfaces and early intracellular aggregation of bacteria. Cellulose is an extracellular bacterial product that acts as a mechanical and chemical protection [7]. The extracellular matrix of *Salmonella* also contains the O-antigenic capsule (O-Ag-capsule), additional capsular polysaccharides, and other lipopolysaccharides [18].

In several epidemic outbreaks of *Salmonella* in humans, animal feed was identified as the source of the same serovars; however, tracing the infection source back through the food supply chain to the farm of origin is highly intricate because of limited identification of animals and limited farm record-keeping [2]. The “farm-to-fork” *Salmonella* surveillance and control system in Sweden recognizes the importance of each step in the feed-animal-food-human chain, and this integrated monitoring proved to be highly successful in reducing the incidence of human salmonellosis [2,19]. In our country, the surveillance of feed factory environments and bacterial contamination of animal feed is still undeveloped and inadequately integrated into the programs for monitoring microbial contamination of products of animal origin for human consumption and the occurrence of human alimentary infections.

## Conclusions

The identification of the same clone of *S. Montevideo* and *S. Infantis*, as well as the weak genetic diversity of *S. Tennessee*, which have been repeatedly isolated in animal feed samples from three production plants, indirectly suggests the possibility of their persistence in feed factory environments. This theory is furthermore supported by the expression of the *rdar* morphotype, which reflects the multicellular behavior pattern of *Salmonella* in the investigated serovars. Common disinfection protocols proved inadequate in successfully eliminating bacteria organized in a biofilm, including *Salmonella* spp. [9,18]. Thus, the programs for controlling *Salmonella* in animal feed have to encompass not only the final products, but the entire production chain. Hazard analysis at critical control points and an effective sanitation program are essential in order to ensure that the processing line is not contaminated with *Salmonella* [20].

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