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Detection of a *Salmonella enterica* Serovar California Strain Spreading in Spanish Feed Mills and Genetic Characterization with DNA Microarrays

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We performed an epidemiological study on *Salmonella* isolated from raw plant-based feed in Spanish mills. Overall, 32 different *Salmonella* serovars were detected. Despite its rare occurrence in humans and animals, *Salmonella enterica* serovar California was found to be the predominant serovar in Spanish feed mills. Different typing techniques showed that isolates of this serovar were genetically closely related, and comparative genomic hybridization using microarray technology revealed 23 *S. enterica* serovar Typhimurium LT2 gene clusters that are absent from serovar California.

Salmonella is one of the major bacterial agents that cause foodborne infections in humans worldwide (9). The principal source of human *Salmonella* infection is contaminated food of animal origin, and animal feed is one source of *Salmonella* for food-producing animals (19). The most prevalent serovars detected in feed products usually are not the same as those that cause disease in humans or animals, possibly because different strains survive in different environments (12). Nevertheless, feeds have been responsible for the infection of poultry with multidrug-resistant nontyphoid *Salmonella* in several industrialized countries (10, 19). Due to the possible importance of contamination in feed and the fact that information about feed as a gate for microbial entrance to the food chain is still lacking, we have analyzed the *Salmonella* isolated from feed mills in different regions of Spain.

For this study we used 231 isolates of *Salmonella enterica* obtained from raw feed of plant origin between May 1999 and May 2001 and 5 *Salmonella* strains supplied by the National Veterinary Laboratory of Spain as controls (7). The isolates were identified by conventional biochemical methods and serotyped at the National Microbiology Laboratory for *Salmonella* of Spain. We distinguished 32 different *Salmonella enterica* serovars (Table 1). We detected some feed-adapted *S. enterica* serovars (such as Senftenberg and Ohio) and *S. enterica* serovars implicated in infections (such as Enteritidis and Typhimurium), but the most prevalent *S. enterica* serovar was California (45% of all isolates), a serovar infrequently detected in animal and human infections.

The antimicrobial susceptibilities of representative strains were determined by the disk diffusion technique according to National Committee for Clinical Laboratory Standards (NCCLS) standards, using ampicillin, amoxicillin-clavulanic acid, nalidixic acid, ciprofloxacin, chloramphenicol, co-trimoxazole, gentamicin, and tetracycline antimicrobial disks (Sanofi Diagnostics Pasteur, Marnes la Coquette, France). The strains were highly susceptible to all tested antimicrobial agents, even those serovars that were occasionally associated with multidrug resistance, such as *S. enterica* serovar Typhimurium or subsp. I 4,5,12:i:− strain (4). This lack of resistance could be related to an absence of antimicrobial pressure in a natural environment.

Due to the importance of the presence of *S. enterica* serovar California in Spanish feed mills, we have analyzed this serovar by epidemiological genotyping methods, including plasmid and pulsed-field gel electrophoresis (PFGE) profiling and comparative genomic hybridization using a *Salmonella*-specific microarray. Plasmid DNA was isolated with the Qiagen plasmid purification kit (Qiagen, Hilden, Germany) and subsequently digested with two different restriction enzymes (*Taq*I and *Hin*dIII). Most (91%) of the *Salmonella* serovar California strains showed the same plasmid profile characterized by a plasmid of approximately 3.5 kb in size. Two strains had two additional genetic elements of 10 and 20 kb, and two strains contained no plasmids. Macrorestriction by PFGE was performed as previously described (5) using restriction endonucleases *Spe*I and *Xba*I (Amersham Pharmacia Biotech, Buckinghamshire, England). Thiourea was added to the electrophoresis buffer to minimize degradation (11, 18). PFGE results were interpreted visually according to published guidelines (20, 21).

The PFGE typing data with *Spe*I and *Xba*I restriction enzymes showed that the *Salmonella* serovar California strains detected were closely related. The *Spe*I PFGE patterns of all the *Salmonella* serovar California strains were grouped into one type called S, which was subdivided into four subtypes (S1 to S4) with only one restriction fragment difference. The obtained *Xba*I patterns were also categorized in one type, called

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TABLE 1. *Salmonella* serovars isolated in feed from mills in different regions of Spain between May 1999 and May 2001

Salmonella serovar	No. of strains isolated in different regions of Spain			Total no. of strains
	Northwest	Northeast	Southeast	
California	10	94		104
Lexington	3	9	6	18
Enteritidis	1	13		14
Poona	1	11		12
Llandoff	6	4		10
Anatum	4	2	3	9
Mbandaka		1	8	9
Derby	2	4		6
Tennessee	3	1	2	6
Kentucky			4	4
Rissen		4		4
Schwarzengrund		4		4
Senftenberg		4		4
Serotype I 3,10:−:1,6	1	1	1	3
Tilburg		3		3
Typhimurium		3		3
Serotype I 4,12:−:−		2		2
Montevideo	2			2
Other serovars[a]	1	10	3	14
Total	34	170	27	231

[a] Agona, Cubana, Essen, Hadar, Majdoiro, Mikawasima, Muenchen, Ndolo, Ohio, Oranienburg, subsp. I 4,12:g,m,t:z_{39}, subsp. I 3,10:−:−, subsp. I 4,12:−:1,2, and subsp. I 4,5,12:i:−.

X, and seven subtypes (X1 to X7), with three or less different fragments (Fig. 1). The combination of the data from both enzymes did not improve the discrimination obtained with *Xba*I. Plasmid content and PFGE data suggested that a single strain was spreading in mills located in several regions of the country. As previously suggested (10), we hypothesized that this spread could be related to the import of *Salmonella*-contaminated plants, as there was no evidence for raw feed exchange between mills.

We detected a subtle evolution of the *Salmonella* serovar California genotypes over time. Some PFGE patterns were prevalent at the beginning of the study and were replaced by others during the study period (Fig. 1). At the beginning of our study (from May 1999 to August 2000), the prevalent PFGE profiles were S1 and X1, which were later replaced by S2 and S3 (for the *Spe*I enzyme) and X4, X5, and X6 (for the *Xba*I enzyme). Heir et al. showed that a change in the season resulted in replacement of a prevalent *Salmonella* macrorestriction profile by a very different one in Norwegian patients (8). However, in this study, patterns S1 and X1 were very similar to their derivative subtypes, supporting a genetic evolution hypothesis and suggesting a selection of the best-adapted strains to the ecological niche, as described for *Pseudomonas fluorescens* by Rainey and Travisano (17).

One representative strain of the spreading *S. enterica* serovar California was hybridized to a previously described *Salmonella*

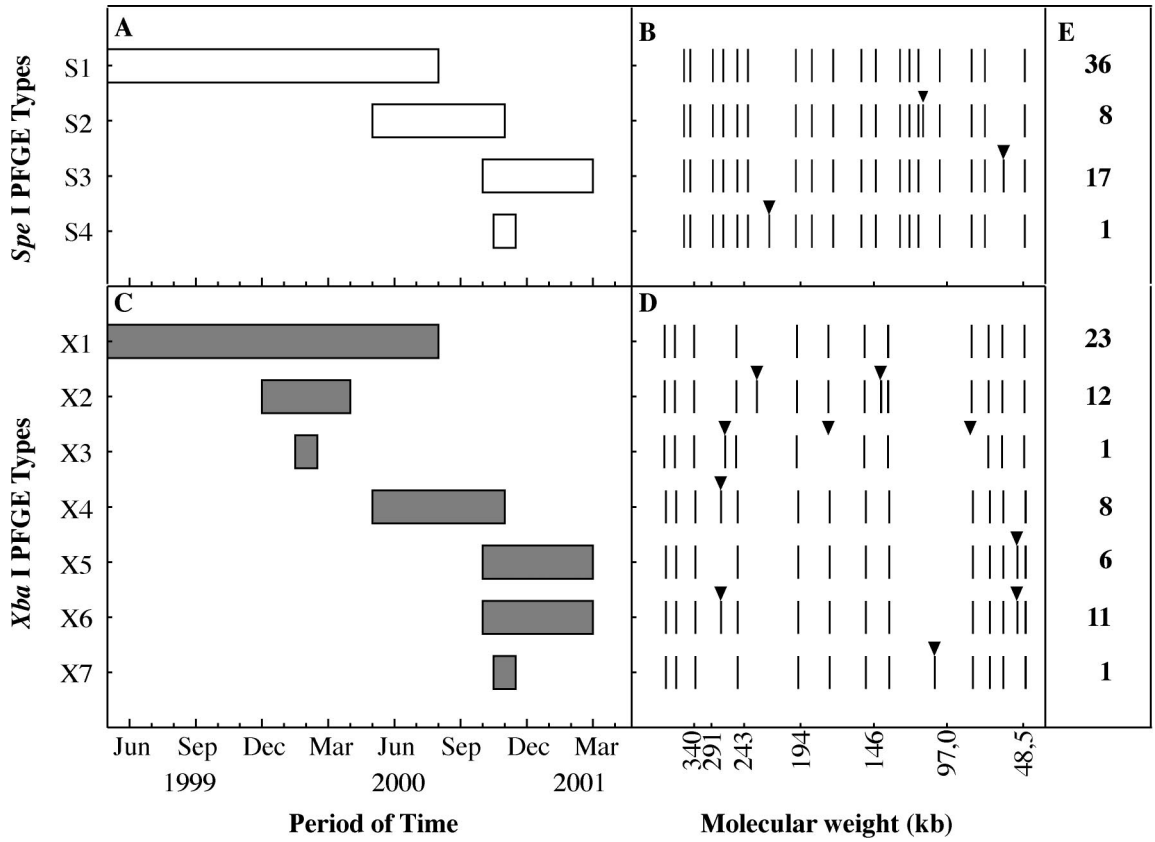

FIG. 1. PFGE analysis of *S. enterica* serovar California strains identified in Spanish feed mills. Distribution of *Spe*I (A) and *Xba*I (C) PFGE profiles over time and the *Spe*I (B) and *Xba*I (D) PFGE profiles obtained (black arrowheads point to differences from profile S1 or X1). (E) Number of isolates for each PFGE pattern.

TABLE 2. Gene clusters in the *Salmonella* serovar Typhimurium LT2 strain absent from the *Salmonella* serovar California strain

STM number[a]	Gene[b]	Function(s)
STM0030—0037		Putative genes
STM0290—0292		Putative genes
STM0299—0304	*safABCD-ybeJ-sinR*	Fimbrial operon, transcriptional regulator
STM0325—0334		Putative genes
STM0571—0576		Putative genes
STM0716—0727		Putative genes
STM0854—0860		Putative genes
STM0893—0929		Fels-1 prophage
STM1005—1056		Gifsy-2 prophage
STM1860—1871	*pagOK*	PhoPQ-activated, phage-related, and putative genes
STM2230—2244	*oafA sspH2*	Phage-related genes
STM2584—2636		Gifsy-1 prophage
STM2694—2772	*fljAB-hin*	Fels-2 prophage, phase switching operon
STM3117—3123		Putative genes
STM3251—3256	*agaR*	PTS
STM3277—3278		Putative genes
STM3752—3755	*sugR-rhuM*	ATP binding protein
STM3780—3784		Putative genes
STM3827—3830	*dgoTAKR*	D-Galacturonic acid metabolism
STM3844—3846		Putative genes
STM4109—4116	*talC-ptsA-frwBCD-pflCD*	PTS
STM4488—4498		Putative genes
STM4523—4528	*yjiW-hsdSMR-mrr*	Restriction and modification system

[a] STM, *Salmonella* serovar Typhimurium complete genome sequences.
[b] If known.

DNA microarray (6, 13, 15, 16), which contains almost all the coding DNA sequences (CDSs) from *S. enterica* serovar Typhimurium LT2 and the pSLT virulence plasmid. Overall, 311 CDSs of the 4,338 chromosomal open reading frames represented on the microarray were predicted to be absent or diverged and 280 CDSs were classified as uncertain. Table 2 summarizes regions of two or more genes of the serovar Typhimurium LT2 genome that are apparently absent from the serovar California strain investigated. Of these 23 gene clusters, 12 contained genes with no assigned function. In addition, the *dgo* operon involved in D-galacturonic acid metabolism, two phosphotransferase systems (PTS), some *phoPQ*-activated genes, a cluster containing phage remnants, one of the fimbrial operons (*saf*), and all four active prophages from the serovar Typhimurium LT2 genome were absent from serovar California. The serovar California isolate also contained a different *hsdSMR* restriction and modification system and lacked all pSLT genes, including the *spv* gene cluster, frequently associated with virulence (2). The major *Salmonella* pathogenicity islands and most of the flagellar genes appeared to be present in *Salmonella* serovar California, but the status of the gene encoding the major flagellin, *fliC*, was uncertain. Reliable hybridization of DNA to the target gene on the array occurs only if sequence identities are at least 97% over one 100-bp window within a gene (14) or if there is >90% DNA sequence identity over the entire gene. A previously sequenced *Salmonella* serovar California *fliC* gene (GenBank accession no. U05296) and the *Salmonella* serovar Typhimurium LT2 *fliC* gene share only a 64.5% similarity using the Matcher tool available at the Institute Pasteur website (http://bioweb.pasteur.fr). Therefore, the microarray correctly reported the gene to be divergent. The apparent absence of the highly conserved regulatory *hin* gene involved in flagellar phase switching explained the monophasic state of the California strain.

S. enterica serovar California has been isolated from cases of human gastroenteritis (1, 3). However, the frequency of its involvement in human disease is insignificant compared to its apparent prevalence in feed. Therefore, it seems likely that neither humans nor the farm animals are the natural host for serovar California and that its natural host is yet to be elucidated. Alternatively, this strain may normally remain completely asymptomatic in a human or farm animal host.

In summary, we have detected the spread of a single *Salmonella* serovar California strain in raw plant-based feed samples from mills located in different regions of Spain. PFGE studies suggested genetic adaptation of the strain over time, while microarray analysis was used to further characterize the genetic repertoire present in this serovar. Epidemiological surveillance for *Salmonella* in local or imported supplies that will become part of animal feeds should be encouraged in order to prevent the introduction of this microorganism to animals and humans through this route.

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