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Molecular Analyses of *Salmonella enterica* Isolates from Fish Feed Factories and Fish Feed Ingredients

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Isolates of the most commonly observed salmonella serovars in Norwegian fish feed factories from 1998 to 2000 (*Salmonella enterica* serovar Agona, *S. enterica* serovar Montevideo, *S. enterica* serovar Senftenberg, and *S. enterica* serovar Kentucky) were studied by pulsed-field gel electrophoresis (PFGE) and plasmid profile analysis and compared to isolates of the same serovars from fish feed ingredients, humans, and other sources (a total of 112 isolates). Within each serovar, a variety of distinct PFGE types (with similarity levels less than 90%) were observed in the feed ingredients and other sources, while only two distinct types of each serovar were identified in the factories. The combined results of PFGE and plasmid analyses showed that each factory harbored only a few *S. enterica* clones. Some of these clones persisted for at least 3 years in the factories, indicating that there was long-lasting contamination probably due to inadequate decontamination procedures.

The occurrence of *Salmonella* spp. in feed and feed ingredients is a well-recognized problem worldwide, and feed ingredients are believed to represent a major risk of salmonella contamination in feed factories (9, 11–13, 19). In addition, wild birds, rodents, and insects may carry salmonella, but the significance of these species as sources of contamination in factories is unclear (1, 3, 10, 11).

In Norwegian fish feed factories, strict control measures are used to ensure that the fish feed produced is not contaminated with *Salmonella* spp. The feed factories are required to have in place internal controls based on the hazard analysis by critical control (HACCP) system, in addition to a surveillance program run by official authorities. By using these controls, *Salmonella* spp. were identified in four feed factories. In all four factories, one or two serovars were repeatedly isolated over a period of up to 10 years. It was not known whether the presence of these bacteria was due to a few long-lasting contaminants or to several successive contamination events. We suggest that if long-lasting contaminants are present, the routines for decontamination have been inadequate and that if several succeeding contamination events have occurred, improving the routines to prevent bacteria from entering the factories is probably more important.

The objective of this study was to examine these questions by studying the clonal relationships of the salmonella serovars isolated most frequently from the factories and from fish feed ingredients over a period of time. Pulsed-field gel electrophoresis (PFGE) and plasmid profile typing were used, as these methods have been suggested for differentiation of salmonellas (18) due to their discriminatory power and due to successful application in previous epidemiological investigations (2, 7, 16). To evaluate the epidemiological significance of the PFGE and plasmid profiles obtained, strains of the same serovars isolated from human cases and other sources were also studied.

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MATERIALS AND METHODS

Salmonella isolates. From 1998 to 2000, nine different serovars of *Salmonella enterica* were identified in four fish feed factories by using the internal control systems of the factories, and 14 different serovars were identified in samples of

TABLE 1. Salmonella isolation with confirmed serovars during 1998 to 2000 for fish feed factories A to D and for fish feed ingredients (mainly fish meal)

Salmonella serovar	No. of positive samples in fish feed factories				No. of positive batches of fish feed ingredients
	Factory A	Factory B	Factory C[a]	Factory D	
Agona	32[b]	31[b]			
Montevideo	11[b]		9		2
Kentucky				18[d]	
Senftenberg				37[d]	4
Livingstone	5[c]				1
Tennessee	1				
Worthington	1				
Anatum		2			1
Enteritidis				1	
Bere					1
Berta					1
Cerro					4
Havana					1
Lexington					1
Lille					1
Ohio					1
Schwarzengrund					1
sp. (6,7:b:-)					1
Stanley					1

[a] Data available only for 1999 and 2000.
[b] Isolated in 1998, 1999, and 2000.
[c] Isolated in 1998 and 1999.
[d] Isolated in 2000.

TABLE 2. Number of *S. enterica* serovar Agona isolates with each PFGE type and plasmid profile[a]

Source of isolates	No. of isolates with the following PFGE types and plasmid profiles							
	Type AR, profile 0	Type A1			Type A2		Type A3, profile aa	Type A4, profile 0
		Profile 0	Profile aa	Profile ab	Profile 0	Profile aa		
Factory A (1998)			8			1		
Factory A (1999)		1	5				1	
Factory A (2000)			1			4		
Factory B (1998)			8		1			
Factory B (1999)					1			
Factory B (2000)			2	1	3	1		
Fish meal (1992, 1995)								
Other feed mill (1998)								
Human feces								1
Sewage sludge								
National reference strain	1							
Total	1	1	24	1	5	6	1	1

[a] Profile 0 indicates that there are no plamids. Profile ab has two of the three plasmids of profile aa, and profile ac has the three plasmids of profile aa in addition to three other plasmids. All plasmids are smaller than 35 MDa.

fish feed ingredients by using official and private control systems (Table 1). In the fish feed factories, 90% of the isolates belonged to *S. enterica* serovar Agona, *S. enterica* serovar Montevideo, *S. enterica* serovar Senftenberg, and *S. enterica* serovar Kentucky. A total of 69 isolates of these four serovars obtained from fish feed factory environments and feed batches were included in the study. In addition, all seven available isolates of the same serovars obtained from 1998 to 2000 from domestic and imported fish feed ingredients (mainly fish meal) were included in the study, together with four feed ingredient isolates obtained in previous years (*S. enterica* serovar Agona isolates from 1992 and 1995 samples, an *S. enterica* serovar Montevideo isolate a from 1996 sample, and an *S. enterica* serovar Senftenberg isolate from a 1997 sample). Three of these isolates were obtained from ingredients that were brought to the premises of a fish feed factory before they were tested. The remaining eight isolates originated from fish meal batches that were tested before they entered any feed factory; since they were positive, these batches were not allowed into any of the factories.

To study the degree of polymorphism of the PFGE types and plasmid profiles of the different serovars used, 28 selected strains belonging to the same serovars that were isolated from other sources during 1996 to 2000 were obtained from

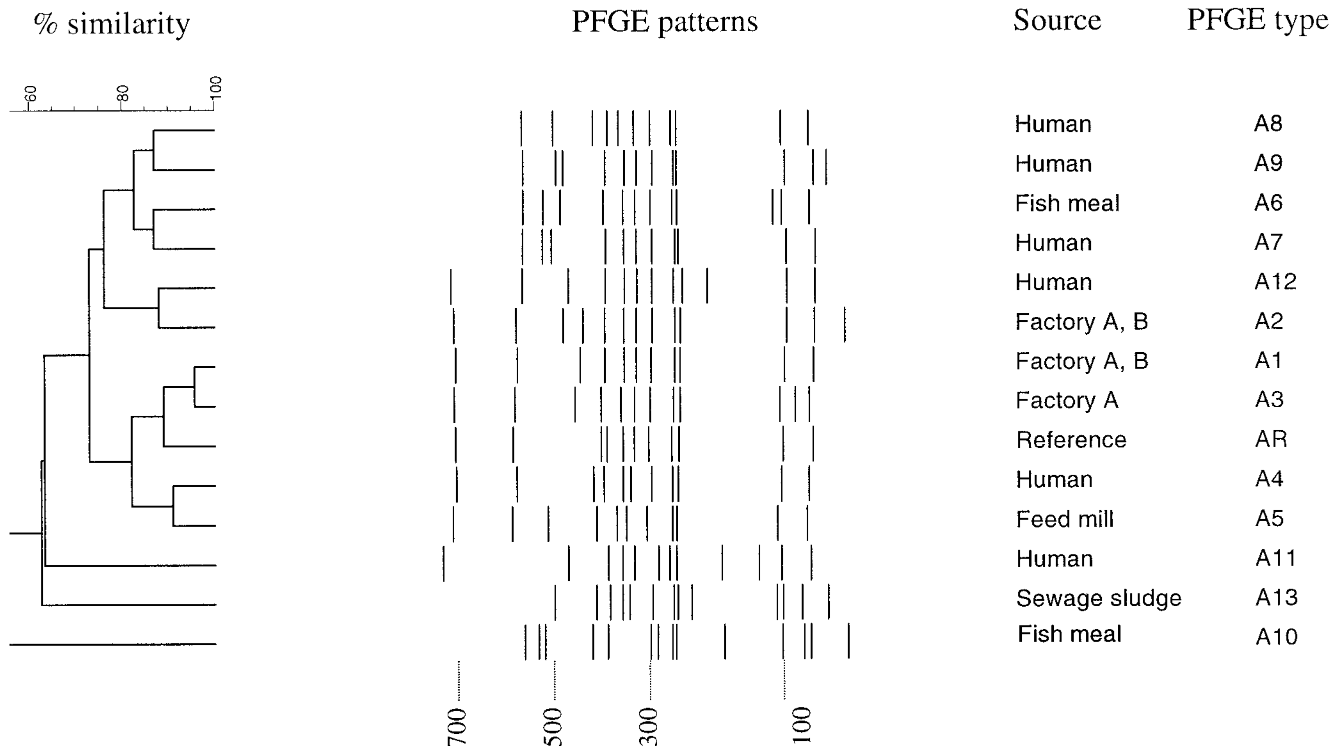

FIG. 1. Dendrogram of *S. enterica* serovar Agona isolates based on PFGE (*Xba*I) fragment patterns, corresponding PFGE type designations, and sources of isolation.

TABLE 2—*Continued*

No. of isolates with the following PFGE types and plasmid profiles											Total
Type A5			Type A6, profile 0	Type A7, profile 0	Type A8, profile 0	Type A9, profile 0	Type A10, profile 0	Type A11, profile 0	Type A12, profile 0	Type A13, profile 0	
Profile 0	Profile aa	Profile ac									
											9
											7
											5
											9
											1
											7
			1				1				2
		1									1
1	1			1	1	1		1	1		8
											1
											1
1	1	1	1	1	1	1	1	1	1	1	51

the strain collections of the National Veterinary Institute and the Norwegian Institute of Public Health. There had been no known epidemiological contact between any of the sources of these isolates or between the sources and the fish feed factories and fish feed ingredients tested.

All strains ($n = 108$) were isolated at private or official laboratories, and the identities were verified at the National Salmonella Reference Laboratory (Norwegian Institute of Public Health). In addition, national reference strains for all four serovars were included in the study. These strains were originally obtained from L'Institut Pasteur, Paris, France.

PFGE. Genomic DNA preparation, restriction enzyme digestion with *Xba*I, and PFGE were performed as previously described (4). A lambda ladder PFGE marker (New England BioLabs, Beverly, Mass.) was used as a size marker. Restriction patterns that differed by one or more bands were given different designations, each of which consisted of an uppercase letter (indicating the serovar) and a number (the letter used for reference strain patterns was R). Images of PFGE gels obtained by using GelDoc 2000 and Quantity One software (Bio-Rad, Hercules, Calif.) were saved in TIFF format and were transferred to the GelComparII software (Applied Maths, Kortrijk, Belgium) for computer-assisted analysis. Similarity between fingerprints was determined by using the Dice coefficient and a band position tolerance of 1%. Fragments in the range from 48.5 to 776 kbp were included. Dendrograms were generated by the unweighted pair group method with arithmetic averages.

Plasmid profile analysis. Separation of plasmid DNA and plasmid profile analysis were performed as described elsewhere (12). Plasmid molecular masses were determined by electrophoresis; plasmids having known molecular masses from *Escherichia coli* 517 were included (8). Plasmid profile designations consisted of two lowercase letters, the first of which indicated the serovar.

RESULTS

Only two distinct PFGE types (similarity levels, <75%) for each serovar were identified for isolates from the fish feed factories; these PFGE types were *S. enterica* serovar Agona types A1 and A2, *S. enterica* serovar Montevideo types M1 and

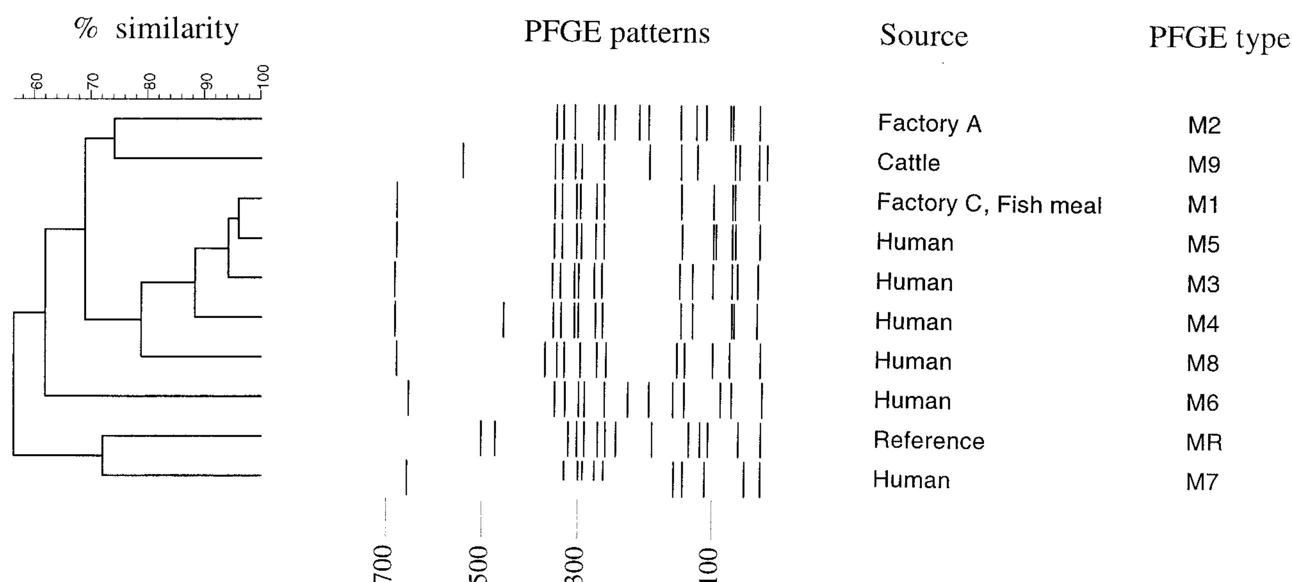

FIG. 2. Dendrogram of *S. enterica* serovar Montevideo isolates based on PFGE (*Xba*I) fragment patterns, corresponding PFGE type designations, and sources of isolation.

TABLE 3. Number of *S. entarica* serovar Montevideo isolates with each PFGE type and plasmid profile[a]

Source of isolates	No. of isolates with the following PFGE types and plasmid profiles					
	Type MR, profile 0	Type M1				Type M2, profile 0
		Profile 0	Profile ma	Profile mb	Profile mc	
Factory A (1998)						4
Factory A (1999)						1
Factory A (2000)						2
Factory C (1999)			2			
Factory C (2000)		5		1		
Fish meal tested at factory C in 2000		1				
Fish meal (1996, 2000, 2000)		3				
Other feed mill (1998)					1	
Human feces						
Cattle feces						
National reference strain	1					
Total	1	9	2	1	1	7

[a] Profile 0 indicates that there are no plasmids. Plasmid profiles mg and mh each have one plasmid that is larger than 35 MDa. All other plasmids are smaller than 35 MDa.

M2, *S. enterica* serovar Senftenberg types S1 and S2, and *S. enterica* serovar Kentucky types K1 and K4 (Tables 2 to 5 and Fig. 1 to 4). Both *S. enterica* serovar Agona PFGE types and both *S. enterica* serovar Montevideo PFGE types were repeatedly isolated throughout the study period (1998 to 2000). A third *S. enterica* serovar Agona type (type A3) that was 95% similar to type A1 was isolated once in 1999. The correspondence between PFGE type and plasmid profile within factories was 100% for the isolates of *S. enterica* serovar Senftenberg and 90% for the isolates of *S. enterica* serovar Agona and *S. enterica* serovar Kentucky. More than 85% of the *S. enterica* serovar Montevideo isolates from factories lacked plasmids.

The 11 isolates from fish feed ingredients (fish meal, corn gluten) displayed eight different PFGE type-plasmid profile combinations (Tables 2 to 4). Two of the combinations (type M1 and profile 0; type S1 and profile sa) were also identified in factory isolates, whereas none of the combinations was identified for the isolates from other sources. All four *S. enterica* serovar Montevideo isolates from fish meal had the same PFGE type (type M1) and the same lack of plasmids as found in factory C. Furthermore, one of these fish meal isolates originated from a batch of meal that was tested at factory C. An *S. enterica* serovar Senftenberg isolate from a sample of corn gluten tested after arrival at factory D had both the same PFGE type (type S1) and the same plasmid profile (profile sa) as isolates from environmental and feed samples from factory D.

The 32 isolates from humans and other sources (including national reference strains) displayed 30 different PFGE type-

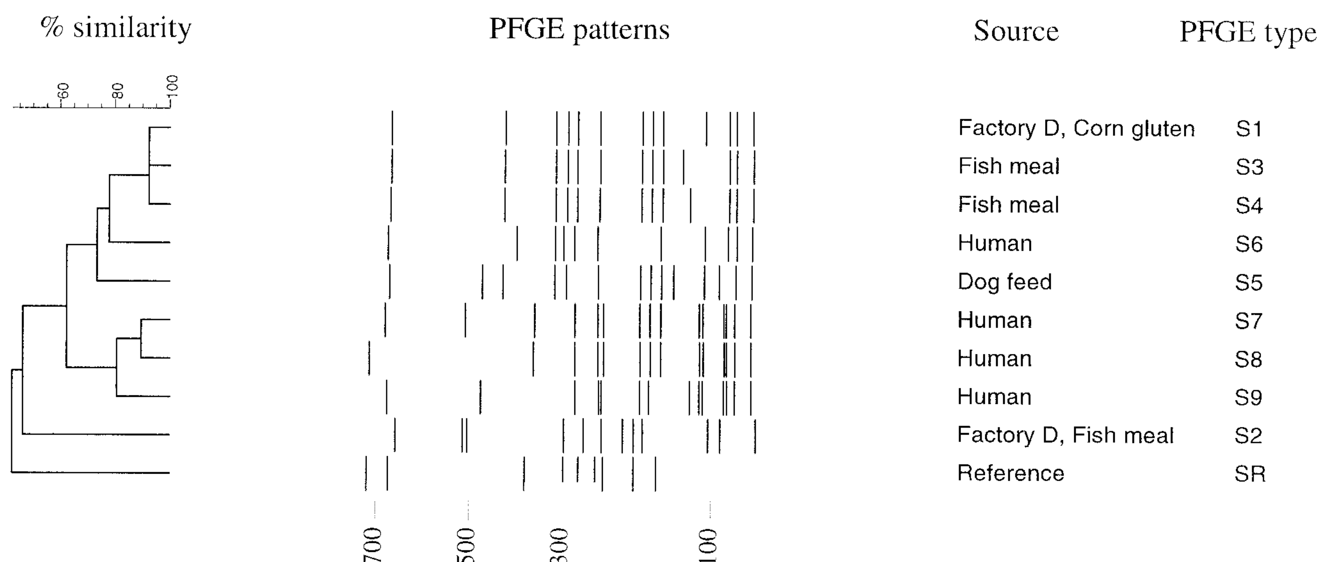

FIG. 3. Dendrogram of *S. enterica* serovar Senftenberg isolates based on PFGE (*Xba*I) fragment patterns, corresponding PFGE type designations, and sources of isolation.

TABLE 3—*Continued*

No. of isolates with the following PFGE types and plasmid profiles								Total
Type M3		Type M4, profile mf	Type M5, profile 0	Type M6, profile 0	Type M7, profile mg	Type M8, profile 0	Type M9, profile mh	
Profile md	Profile me							
								4
								1
								2
								2
								6
								1
								3
								1
1	1	1	1	2	1	1		8
							1	1
								1
1	1	1	1	2	1	1	1	30

plasmid profile combinations (Tables 2 to 5 and Fig. 1 to 4). Only one of these isolates, an *S. enterica* serovar Montevideo isolate from another feed mill, had a PFGE type (type M1) identical to any of the types found in the fish feed factories and fish feed ingredients. However, the feed mill isolate had a distinct plasmid profile (profile mc). Two isolates from human feces had plasmid profiles identical to profiles found in the factories but had different PFGE types (*S. enterica* serovar Agona plasmid profile aa and *S. enterica* serovar Kentucky profile ka).

DISCUSSION

Over a 3-year period, only two distinct PFGE types were observed for members of each of the four most prevalent salmonella serovars isolated from fish feed factories. This is in contrast to the relatively large number of PFGE types obtained when epidemiologically unrelated isolates belonging to each serovar were subjected to cluster analyses. Distinct PFGE types with similarity levels of less than 90% were obtained for nearly all of the epidemiologically unrelated isolates studied, illustrating and confirming the discriminatory power and suitability of PFGE for typing these salmonella serovars (6, 15, 17). Identical PFGE types could in certain cases be differentiated by plasmid content, but within each factory only single isolates having each PFGE type displayed variations in the plasmid profile. This may have been due to instability and/or mobility of plasmids, as observed by other workers (5, 14). Therefore, the overall correspondence between PFGE type and plasmid profile over time indicates that the salmonella bacteria isolated from the factories originated from a few distinct clones.

Several investigations have shown that fish meal, as well as raw material of vegetable origin, can be contaminated by *Salmonella* spp. (3, 9,19). It is difficult to observe a direct epidemiological relationship between ingredient batches that are salmonella positive and contamination in factories, because such batches are normally not allowed on factory premises. However, our analyses showed that a batch of corn gluten was the probable source of *S. enterica* serovar Senftenberg contam-

TABLE 4. Number of *S. enterica* serovar Senftenberg isolates with each PFGE type and plasmid profile[a]

Source of isolates	No. of isolates with the following PFGE types and plasmid profiles												Total
	Type SR, profile sr	Type S1, profile sa	Type S2		Type S3, profile 0	Type S4		Type S5, profile 0	Type S6, profile 0	Type S7, profile sd	Type S8, profile se	Type S9, profile sf	
			Profile 0	Profile sb		Profile 0	Profile sc						
Factory D (2000)		3		3									6
Corn gluten tested at factory D (2000)		1											1
Fish meal tested at factory D (1998)						1							1
Fish meal (1997, 2000, 2000)			1		1		1						3
Commercial dog feed								1					1
Human feces or urine									1	1	1	1	4
National reference strain	1												1
Total	1	4	1	3	1	1	1	1	1	1	1	1	17

[a] Profile 0 indicates that there are no plasmids. All plasmids are smaller than 35 MDa.

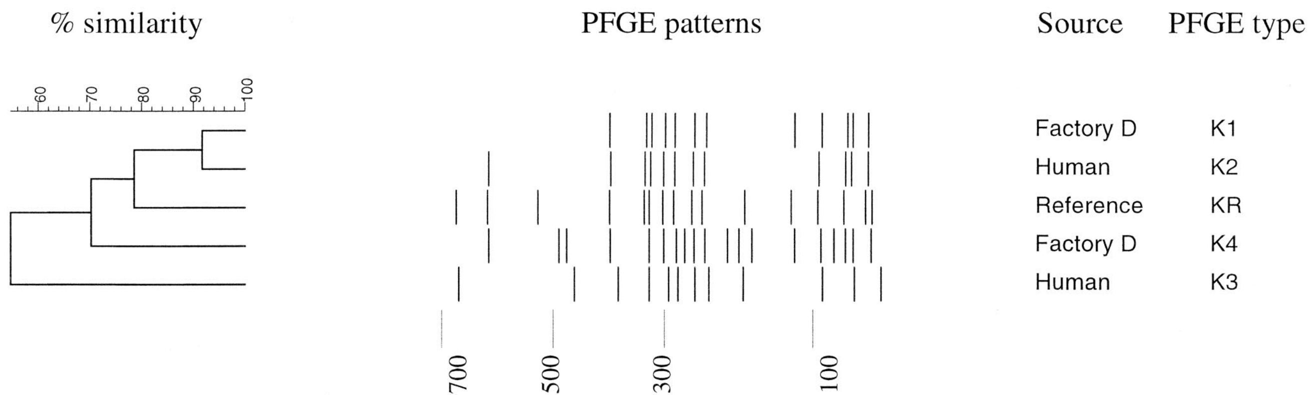

FIG. 4. Dendrogram of *S. enterica* serovar Kentucky isolates based on PFGE (*Xba*I) fragment patterns, corresponding PFGE type designations, and sources of isolation.

ination in one of the factories (factory D). Furthermore, the *S. enterica* serovar Montevideo type in factory C (type M1) was also identified in several samples of fish meal. Therefore, our results support the presumption that fish feed ingredients may represent a risk of introducing salmonella into fish feed factories.

The fish feed factories buy fish meal and other ingredients from a relatively large number of suppliers. In ingredients tested in Norway, we identified 14 different serovars during 1998 to 2000. Furthermore, the 11 isolates studied displayed eight different PFGE type-plasmid profile combinations. This relatively large variation in salmonella strains found in ingredients makes it less likely that the factories have repeatedly received the same few salmonella clones from the ingredients, although this possibility cannot be excluded in the case of factory C. We therefore suggest that salmonella clones may have entered the fish feed factories with fish feed ingredients but in most cases have continued to persist in the factories because the decontamination routines in the factories have been inadequate.

Only four isolates with four different PFGE types (types M7, M9, KR, and K4) contained plasmids large enough (>35 MDa) to be detected and included in the analyses of the PFGE data. These four PFGE types were all less than 80% similar to any other PFGE type, indicating that there were differences in chromosomal DNA in addition to the possible differences caused by plasmid DNA. Therefore, the possible presence of plasmids or plasmid fragments in the PFGE gels did not influence the conclusions of the present study.

TABLE 5. Number of *S. enterica* serovar Kentucky isolates with each PFGE type and plasmid profile[a]

Source of isolates	No. of isolates with the following PFGE types and plasmid profiles						Total
	Type KR, profile kr	Type K1		Type K2, profile ka	Type K3, profile 0	Type K4, profile kb	
		Profile 0	Profile ka				
Factory D (2000)		1	8			1	10
Human feces				1	2		3
National reference strain	1						1
Total	1	1	8	1	2	1	14

[a] Profile 0 indicates that there are no plasmids. Plasmid profiles kr and kb each have two plasmids that are larger than 35 MDa. All other plasmids are smaller than 35 MDa.

In conclusion, this study revealed the long-lasting persistence of certain salmonella clones in fish feed factories. The clones may persist on production equipment or in other parts of the factory environment that are difficult to decontaminate. In addition, the possibility that resident birds, small rodents, or insects are carriers cannot be eliminated.

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