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Longitudinal Study of Shiga Toxin-Producing *Escherichia coli* Shedding in Sheep Feces: Persistence of Specific Clones in Sheep Flocks▽

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To provide information on the persistence and maintenance of colonization with Shiga toxin-producing *Escherichia coli* (STEC) in sheep, pulsed-field gel electrophoresis analysis of STEC isolates ($n = 145$) belonging to serogroups O5, O91, and O146 from 39 healthy animals was performed in a 12-month longitudinal study carried out with four sheep flocks. At the flock level as well as the individual-animal level, the same clones were obtained on sampling occasions separated by as much as 11 months.

Shiga toxin-producing *Escherichia coli* (STEC) strains have recently emerged as important food-borne pathogens. Human diseases ranging from mild diarrhea to hemorrhagic colitis and hemolytic-uremic syndrome, typically affecting children, the elderly, and immunocompromised patients, can be caused by STEC (6). Serogroup O157 especially represents a major public health concern worldwide. However, as non-O157 STEC strains are more prevalent than O157 strains in meat-producing animals and as contaminants in foods, humans are more likely to become exposed to such strains (3, 9, 23), and therefore, non-O157 STEC should not be overlooked in human disease investigations. Although healthy asymptomatic cattle are the best-recognized animal reservoir for STEC strains (5), sheep are an important source of these organisms for humans in some countries (2, 4, 8, 19). The ecology and epidemiology of STEC O157 in cattle appear to be very complex, often involving multiple clones on a single farm (14, 17). There is some previously published information about the on-farm persistence of specific clones in different cattle production systems (13, 15, 18, 21), including information about persistence in individual animals in a few cases. Nevertheless, apart from the findings of a study of STEC O157 (12), little is known about the natural colonization of sheep with STEC over long time periods and the ecology of STEC strains in sheep flocks. A longitudinal study was conducted to provide information on the persistence and maintenance of colonization with non-

TABLE 1. PFGE types and persistent clones among non-O157 STEC isolates

Serogroup	No. of isolates	PFGE types (no. of isolates)	No. of types	Persistent clones[a] (no. of types)	No. of clones
O146	50	A1 (10), A2 (1), A3 (1), A4 (6), A5 (8), A6 (1), A7 (1), A8 (1), A9 (2), A10 (1), A11 (1), A12 (1), A13 (1), A14 (1), A15 (5), A16 (2), A17 (3), A18 (1), A19 (1), A20 (1), A21 (1)	21	AI (7), AII (2), AIII (1), AIV (1), AV (2)	5
O91	64	B1 (9), B2 (2), B3 (1), B4 (1), B5 (2), B6 (1), B7 (1), B8 (2), B9 (1), B10 (1), B11 (1), B12 (2), B13 (1), B14 (1), B15 (1), B16 (1), B17 (4), B18 (2), B19 (4), B20 (4), B21 (1), B22 (1), B23 (1), B24 (3), B25 (1), B26 (1), B27 (1), B28 (3), B29 (1), B30 (2), B31 (1), B32 (3), B33 (1), B34 (1), B35 (1)	35	BI (4), BII (3), BIII (2), BIV (2), BV (3), BVI (2), BVII (4), BVIII (6)	8
O5	31	C1 (7), C2 (2), C3 (1), C4 (1), C5 (1), C6 (1), C7 (4), C8 (1), C9 (2), C10 (1), C11 (1), C12 (1), C13 (2), C14 (1), C15 (1), C16 (1), C17 (1), C18 (1), C19 (1)	19	CI (6), CII (5), CIII (1)	3
Total	145		75		16

[a] Based on the criteria that isolates belong to the same serogroup and have PFGE patterns differing by three bands or fewer.

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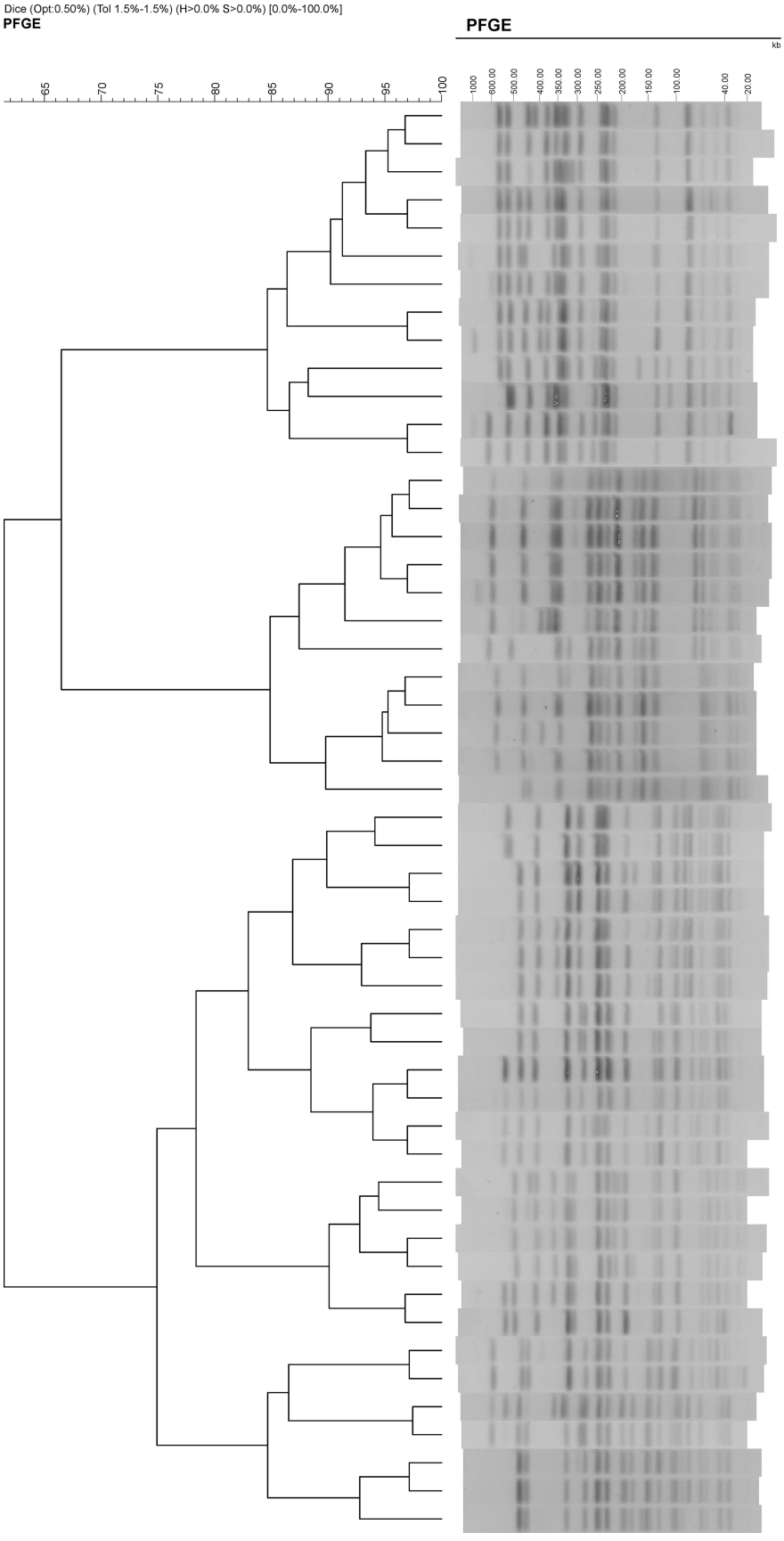

Serogroup	PFGE type	Clone
O146	A4	AI
O146	A1	AI
O146	A2	AI
O146	A6	AI
O146	A5	AI
O146	A15	AI
O146	A7	AI
O146	A10	AII
O146	A12	AII
O146	A17	AIV
O146	A16	AIII
O146	A19	AV
O146	A18	AV
O5	C4	CI
O5	C5	CI
O5	C6	CI
O5	C1	CI
O5	C2	CI
O5	C3	CI
O5	C13	CIII
O5	C10	CII
O5	C7	CII
O5	C9	CII
O5	C8	CII
O5	C12	CII
O91	B5	BI
O91	B6	BI
O91	B2	BI
O91	B1	BI
O91	B9	BII
O91	B8	BII
O91	B10	BII
O91	B21	BVI
O91	B20	BVI
O91	B22	BVII
O91	B23	BVII
O91	B25	BVII
O91	B24	BVII
O91	B29	BVIII
O91	B31	BVIII
O91	B28	BVIII
O91	B30	BVIII
O91	B32	BVIII
O91	B33	BVIII
O91	B14	BIV
O91	B15	BIV
O91	B13	BIII
O91	B12	BIII
O91	B18	BV
O91	B17	BV
O91	B19	BV

TABLE 2. Temporal isolation of non-O157 STEC clones at the flock level

Flock	Clone isolated in (mo/yr)[a]:											
	11/03	12/03	1/04	2/04	3/04	4/04	5/04	6/04	7/04	8/04	9/04	10/04
1	AI	AI	AI	AI	AI	AI	AI	AI	AI	NS	AI	AI
								AIV	AIV	NS	AIV	
	BI	BI	BI	BI	BI	BI	BI	BI	BI	NS	BI	
			BII	BII	BII		BII			NS		
							CII	CII	CII	NS	CII	CII
2									AI			AI
		BIII	BIII					BIII				
		BV	BV		BV	BV	BV		BV			
	BVI	BVI			BVI	BVI						
		BVII	BVII		BVII					BVII		
	CI		CI	CI	CI		CI	CI	CI		CI	CI
	CII					CII	CII	CII				
3		AI	AI	AI	NS	AI			AI	NS	AI	
			AIII	AIII	NS					NS		
		AV	AV		NS					NS		
					NS	BIV				NS	BIV	
	BVIII	BVIII	BVIII	BVIII	NS	BVIII	BVIII	BVIII		NS		BVIII
4		NS				AII					AII	
		NS			CI	CI	CI		CI			
		NS					CIII		CIII			

[a] NS, no sample from the flock was obtained on that occasion. An empty cell indicates that STEC strains were not isolated from the premises or that the isolates obtained were genetically unrelated to the persistent clones.

O157 STEC at the flock level as well as the individual-animal level in the context of a typical sheep flock in southwest Spain.

The longitudinal study was conducted with four epidemiologically unrelated sheep flocks in southwest Spain and started in November 2003. During the first sampling visit, 12 ewes around 1 year of age in each flock were randomly selected for sampling, and subsequent monthly sampling visits were carried out until October 2004. On each sampling occasion, one sample of rectal feces per animal was collected from selected individuals. Fecal samples were examined for STEC by both phenotypic (Vero cells) and genotypic (PCR) methods as described previously (19). The identification of O antigen in isolates ($n = 521$) was carried out in the Laboratorio de Referencia de *E. coli* (Lugo, Spain) as described by Guinée et al. (10) by using the full range of O antisera for serogroups O1 to O185. The first confirmed colony from a positive sample was selected as representative for that animal and stored at −80°C until further pulsed-field gel electrophoresis (PFGE) analysis. The preparation and XbaI digestion of DNA for PFGE were conducted as described previously (20). Bands below 33.3 kb were ignored in the analysis because of the difficult band-marking procedure for this region. A single-band difference was defined as significant to name the different PFGE types. Nevertheless, epidemiologically related isolates with PFGE patterns differing by three bands or fewer were considered to be of the same clone, since such differences are consistent with a single genetic event, i.e., a point mutation in a restriction site, a deletion, or an insertion, according to Tenover criteria (22).

A total of 145 non-O157 STEC isolates from 39 healthy animals in four sheep flocks were characterized by PFGE (Table 1). This selection covered all isolates belonging to serogroups O5, O91, and O146, which are serogroups frequently represented among ovine STEC strains and associated with human strains that have caused hemolytic-uremic syndrome (4, 19). Overall, STEC O146 strains were detected in 19 (39.6%) of the animals, STEC O91 strains were detected in 26 (54.3%) of the animals, and STEC O5 strains were detected in 10 (20.8%) of the animals sampled. These 145 non-O157 STEC isolates produced 75 different PFGE types (Table 1). However, based on the previously described criteria, five persistent clones (AI to AV) were identified among O146 isolates, eight clones (BI to BVIII) were identified among O91 isolates, and three clones (CI to CIII) were identified among O5 isolates (Fig. 1; Table 1).

Table 2 illustrates the persistence of specific non-O157 STEC clones at the flock level. The maximum number of consecutive sampling occasions on which a single clone was isolated from a flock was 11 (for the AI clone from flock 1). The maximum time between the first and last recoveries of a single clone from a flock with the isolation of the clone during the intervening period was 11 months (for the CI clone from flock 2). The maximum time between the first and last recov-

FIG. 1. Dendrogram generated with InfoQuestFP software showing the PFGE-XbaI digestion types for non-O157 STEC isolates of the same persistent clones. The bands generated were analyzed by using the Dice coefficient and the unweighted-pair group method with arithmetic averages. The scales at the top indicate the similarity indices (in percentages) and molecular sizes (in kilobases).

TABLE 3. Temporal recovery of non-O157 STEC clones isolated from individual animals on two or more occasions

Flock	Animal	Clone isolated in (mo/yr)[a]:											
		11/03	12/03	1/04	2/04	3/04	4/04	5/04	6/04	7/04	8/04	9/04	10/04
1	1-1	AI	AI	AI	AI	AI					NS		
	1-2		BI			BI					NS	BI	
	1-4	BI		BI			BI		BI	BI	NS		
	1-6								AIV	AIV	NS	AIV	
	1-7					BI		BI		BI	NS		
	1-8			AI	AI	AI	AI	AI	AI	AI/CII	NS	CII	
	1-9			BII	BII	BII		BII	NS	NS	NS	NS	NS
	1-10			AI	AI		AI	CII	CII		NS		CII
	1-12			BI	BI			AI	AI	AI	NS	AI	AI
2	2-2							CII	CII				
	2-5	BVI/CII	BVI			BVI	BVI/CII						
	2-8		BV	BV			BV	BV		BV	NS	NS	NS
	2-9		BIII	BIII					BIII				
	2-10			BVII		BVII							
	2-11		BV	BV		BV	BV						
	2-12	CI		BVII/CI	CI	BVII/CI		CI	CI	CI		CI	CI
3	3-1			BVIII		NS	BVIII	BVIII			NS		
	3-3					NS	BIV				NS	BIV	
	3-5				AI	NS	AI				NS		
	3-6			AIII	AIII	NS			BVIII		NS		BVIII
	3-7				BVIII	NS		BVIII	BVIII		NS		
	3-8		AV	AV	NS	NS	NS	NS	NS	NS	NS	NS	NS
	3-9		AI	AI	AI	NS				AI	NS	AI	
	3-10	BVIII	BVIII	AI	AI	NS	AI				NS		BVIII
4	4-2		NS			CI	CI	CI		CI			
	4-9		NS					CIII		CIII			
	4-12		NS				AII					AII	

[a] NS, no sample from the animal was obtained on that occasion. An empty cell indicates that the sample from the animal was negative for STEC or that isolates were genetically unrelated to the persistent clones.

eries of a single clone from a flock without the isolation of the clone during the intervening period was 5 months (for the BVII, BIV, and AII clones from flocks 2, 3, and 4, respectively).

Table 3 illustrates the persistence of specific non-O157 STEC clones at the individual-animal level. The maximum number of consecutive sampling occasions on which a single clone was isolated from an animal was seven (for the AI clone from animal 1-8). The maximum time between the first and last recoveries of a single clone from an animal with the isolation of the clone during the intervening period was 11 months (for the CI clone from animal 2-12). The maximum time between the first and last recoveries of a single clone from an animal without the isolation of the clone during the intervening period was 10 months (for the BVIII clone from animal 3-10).

Previous works from the United States have indicated that individual STEC O157 strains can be isolated for as long as 2 years from some dairy herds (21), for as long as 10 months on cattle ranges (18), and over the entire feeding period on cattle feedlots (13). Other studies from Italy and the United Kingdom have referred to this persistence on cattle farms (7, 15). In the only study performed with sheep, Kudva et al. (12) isolated individual STEC O157 strains from a single flock for as long as 2 months. To our knowledge, our study is the first report of the persistence and maintenance of colonization with non-O157 STEC in sheep flocks. LeJeune et al. (13) suggested that strain type stability observed on dairy farms for periods from months to years may be the result of the persistent infection of individual animals because the rate of animal turnover in these cattle production systems is vastly lower than is typical of feedlots, where the presence of other, nonanimal stable reservoirs is the most probable explanation for this situation. Actually, some studies describing the on-farm persistence of PFGE types over long time periods have also noted this persistence in individual animals (7, 15, 21). In addition, the persistence of some non-O157 STEC clones at the individual-animal level has been reported in a recent study on dairy goats (16).

Small variations in the PFGE types of isolates obtained from the same individuals in experimental and natural infections, similar to the variations observed in all serogroups in this study, have been reported before (1, 11). We are aware that the storage of only one colony per positive animal may have influenced our results, since individual animals may shed multiple strains simultaneously and the spectrum of types changes over time (12). However, in spite of these limitations, we have successfully demonstrated that some non-O157 STEC clones can be isolated from the same sheep flock, as well as the same animal, over a period of at least 11 months and from the same animal on consecutive sampling occasions for as long as 7 months. In our opinion, given the low rate of animal turnover which is typical of these sheep production systems, persistent individual-animal colonization by specific clones is the most probable explanation for the persistence of PFGE types at the flock level over long time periods. Nevertheless, we could not

evaluate a possible role of residual contamination of the sheep flock environment and recycling through the host in this study.

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