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On-Farm Monitoring of Mouse-Invasive *Salmonella enterica* Serovar Enteritidis and a Model for Its Association with the Production of Contaminated Eggs

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Mice (*Mus musculus*) captured in henhouses were assessed for the presence of salmonellae in spleens. Of 621 and 526 spleens cultured during the first and second years of collection, 25.0 and 17.9%, respectively, were positive for *Salmonella enterica* serovar Enteritidis. Contaminated eggs were cultured from nine houses during the first year of sampling, and for eight of these houses, serovar Enteritidis was recovered from the spleens of mice. Rank sum statistical analysis of positive mouse spleens indicated that three overlapping bacterial populations were present. This pattern of infection was repeated when lipopolysaccharide (LPS) variants were used to infect chicks, and the worst infections were associated with isolates producing high-molecular-weight (HMW) LPS. Mouse isolates were capable of producing unprecedented amounts of HMW LPS as indicated by compositional analysis of six isolates that swarmed across 2% agar, which is a type of bacterial migration dependent upon production of HMW LPS. It is suggested that monitoring serovar Enteritidis cultured from the spleens of mice caught on farms will detect strains that are enhanced in their ability to contaminate eggs, in part because they are able to produce HMW LPS.

Salmonella enterica serovar Enteritidis is a major food-borne pathogen in the United States because of its ability to contaminate the internal contents of eggs (16, 23). While there is evidence that contamination of eggs is a sporadic event and that the chance of consuming such eggs is 1 in 10,000 or less (11, 17), the problems caused by this pathogen suggest that risk assessments do not reflect the extent to and rate at which serovar Enteritidis can emerge to increase risk. To investigate how the presence of virulent organisms in the poultry environment results in contamination of eggs and ultimately human outbreaks, it would be ideal to collect samples from chickens in production by using experimental protocols designed to minimize differences between farms. Such field studies are not possible because producers cannot sacrifice birds that have reached maturity, risk interruption of daily routines, or stress flocks by performing sampling procedures. However, the producers whose farms were investigated during this study allowed us to sample resident rodent populations from henhouses because they, too, were concerned that mice infected with serovar Enteritidis were serving as a source of continual reinfection of newly introduced birds. Although the experimental design is not ideal because of constraints, the number of eggs, environmental samples, and mouse spleens cultured and the length of time samples were collected from henhouses yielded valuable information concerning serovar Enteritidis infections of rodent populations residing in henhouses.

During this study, rodent density was approximated by determining for each farm a rodent index (RI) after collection of mice by live trapping following a detailed protocol (*Salmonella enteritidis* Pilot Project: Rodent Evaluation and Inspection Form) (7). The RIs were grouped as follows: 10 or fewer mice per house, RI = 1 (low); 11 to 25 mice, RI = 2 (moderate); and 26 or more mice, RI = 3 (high). After euthanasia of mice, the spleens were removed aseptically and mailed overnight to Southeast Poultry Research Laboratory on ice, where they were cultured within 3 days of receipt as previously described (3, 18). Flock environments were evaluated for the presence of serovar *Enteritidis* by the culture of samples from manure pit and egg-handling equipment as described previously (22). Following positive environmental culture, eggs were collected from a house every 2 weeks over an 8-week period. At each sampling, 1,000 eggs were collected. Eggs were either broken out and placed into sterile plastic bags at the Pilot Project laboratory or transported directly to cooperating laboratories for breakout of egg contents and subsequent culture by using the established pooled-egg method (2, 9). No more than three representative *Salmonella*-suspect colonies per culture set were selected and stabbed into triple sugar iron and lysine iron agar slants. All presumptive *Salmonella* isolates from eggs were sent to the National Veterinary Service Laboratory, Ames, Iowa (NVSL), for serotyping. Estimates of frequency of egg contamination were calculated by previously described methods (21).

Mouse spleens were obtained from late October 1992 until mid-October 1993 during phase I, and only *Mus musculus* mice (house mice) were captured during this time. Capturing of mice was randomized for phase I by sampling as many farms and henhouses as possible. Phase II began in October 1993 and ended in late September 1994, and its purpose was to follow mouse populations and invasive salmonellae in selected houses for an additional year. When a suspect *Salmonella* colony was cultured, three colonies per isolate were reacted with group D1 antiserum (factors 1, 9, and 12; Difco) by slide agglutination,

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TABLE 1. Incidence of *S. enterica* serovar *Enteritidis* in mouse spleens and eggs

Chicken house code	Vaccination status[a]	House environment[b]	RI[c]	No. of spleens sampled		% of positive spleens		Egg pool sampling[d]
				Phase I	Phase II	Phase I	Phase II	
1501	2	2	3.0	16	ND	6.3		ND
1601	2	3	2.3	26	ND	26.9		1 of 3
2000	1	ND	ND	15	ND	33.3		ND
2008	2	2	1.0	12	ND	0		ND
2054	2	2	3.0	12	ND	0		ND
2401	2	3	1.0	14	ND	21.4		1 of 2
2402	2	3	2.0	46	ND	21.7		0 of 1
2603	3	3	3.0	122	48	40.2	8.3	0 of 5
2604	3	3	2.2	96	15	44.8	26.7	1 of 2
2610	3	1	2.1	66	35	25.8	11.4	4 of 6
2701	1	2	0.9	4	1	25.0	0.0	ND
2702	1	2	1.8	15	62	6.7	0.0	1 of 3
2703	1	ND	ND	4	ND	0.0		ND
2704	1	3	1.3	12	ND	0.0		0 of 3
2705	1	ND	ND	5	ND	0.0		ND
2706	1	3	2.3	23	101	0.0	1.0	0 of 4
2707	1	3	2.2	28	74	0.0	0.0	0 of 4
2801	2	1	2.0	26	ND	15.4		3 of 4
2901	2	3	1.3	35	ND	25.7		3 of 3
4001	2	1	1.5	13	ND	0.0		2 of 3
5301	2	1	3.0	31	170	16.1	47.6	3 of 6

[a] 1, vaccinated; 2, not vaccinated; 3, replacement flock vaccinated.
[b] 1, always positive; 2, always negative; 3, mixed results; ND, not done.
[c] Average rodent density per henhouse during phase I. ND, not done.
[d] Eggs cultured in pools of 10 through March 1992 and in pools of 20 thereafter.

and if positive, the isolates were further characterized by using commercial biochemical profiling assays (Enterotube). Isolates that appeared to be salmonellae but were negative for group D1 epitopes were reacted with other O-antigen antisera and with flagellar H-antigen antisera (G complex; Difco) by slide agglutination. Any isolate with biochemical reactions typical of a salmonella, but lacking O-antigen reactivity, was sent to NVSL for identification and for phage typing.

Incidence of serovar Enteritidis, other salmonellae, and contaminants. During phase I, 25% of 621 cultured spleens were positive for mouse-invasive serovar Enteritidis, while during phase II, 17.9% of 526 spleens were positive (Table 1). All serovar group D1 isolates with biochemical reactions appropriate to salmonellae were classified as *S. enterica* serovar Enteritidis, and this designation was substantiated by confirmation at NVSL. Some *Salmonella* isolates were negative for O-antigen epitopes by slide agglutination (1.2%), and 8 of 10 of these isolates were classified as phage type (pt) 23 serovar Enteritidis, while one was pt 24 and one was pt 8. pt 23 and pt 24 are rough phenotypes of serovar Enteritidis and thus have no O antigen, and the one pt 8 coisolated with pt 23, suggesting that it could have been a minority population in a mixed culture. These isolates were positive for the serovar "g,m" flagellar epitope, were motile, and had typical biochemical profiles of salmonellae. One isolate presented a diagnostic problem since it had group D1 epitopes, no "g,m" flagellar epitopes, and atypical biochemical reactions, but no further attempt was made to identify it after it was classified as a nonsalmonella.

The second most frequently isolated salmonella was serovar group B, presumptively *S. enterica* serovar Typhimurium. Group B salmonellae were isolated from 1.45% of spleens obtained during the first year of sampling (phase I) and 1.1% of spleens collected during the second year (phase II). Contaminant organisms were isolated from 3.4% of total spleens, and common contaminants were *Enterobacter cloacae*, *Citrobacter* spp., *Proteus* spp., *Acinetobacter* spp., *Escherichia coli*, and *Klebsiella* spp. One possible *Arizonae* spp. was encountered. Paired control studies indicated that isolation of contaminants from spleens increased when ice was not used during shipping, whereas recovery of serovar Enteritidis from positive houses was unchanged and spleens from historically serovar Enteritidis-negative houses remained negative. Spleens could be stored up to 1 week at 4°C without experiencing a significant drop in viable organisms.

Farm histories and association of mouse-invasive serovar Enteritidis with contaminated eggs. Details of farm history indicated that houses 2604, 2610, and 2702 were associated with human outbreaks while house 5301 was stocked with replacement chicks positive for serovar Enteritidis (Table 1). Some houses were consistently negative or near-negative during both phases (2706 and 2707), while others were consistently positive (2603, 2604, 2610, and 5301). The average RI for houses positive for serovar Enteritidis in spleens during phase I was 2.05, whereas it was 1.88 in negative houses. Vaccination did not always prevent egg contamination (house 2702), and the presence of serovar Enteritidis in spleens did not always correlate with the production of contaminated eggs (houses 2402 and 2603). Environmental sampling results were variable in some houses and frequently failed to correlate with the presence of serovar Enteritidis in spleens or contaminated eggs (houses 1501, 1601, 2604, 2701, 2702, and 2901). However, environmental sampling was the only parameter associated with contaminated eggs in house 4001. The number of mice sampled from this house was small (13 mice) and possibly an inadequate sampling size.

Continued monitoring of select houses during phase II indicated serovar Enteritidis-positive houses 2603, 2604, 2610, 2701, and 2702 all experienced a decline in the percentage of positive mouse spleens. House 5301, which was originally populated with infected pullets, had a higher number of positive mouse spleens the second year.

Analysis of data collected from naturally infected mice suggests there are three serovar Enteritidis populations. The type of statistical analysis used here to analyze batches of data derived from culture of spleens was nonparametric rank sum analysis (15). It was chosen as the most appropriate analytical method because it detects data composed of separate populations, and thus it is a type of analysis that only substantiates large differences within data sets. Rank analysis without summation is a general statistical approach to analyze data that may not conform to parametric assumptions, as evidenced by clustering of data after ranking in order. To do rank sum analysis, data that are tied are summed while their rank orders to other data are maintained by assigning place value. Polynomial analysis is applied to ranked and summed data sets, which generates standard bell curves, and thus it becomes possible to assess the characteristics of multiple populations that are present within a single nonrandom data set.

Generation of random numbers between 0.1 and 0.8, which is the range of positive sample data collected as batches of spleens (Fig. 1), indicated that a normally distributed population of numbers produces an ascending straight line when data are arranged from least to greatest (Fig. 2a). To see if sample batch data appeared to be from a single population, positive batches of spleens collected per farm were ranked from least to greatest by the percentage of positive spleens recovered (Fig. 1; Fig. 2a). Curvolinear analysis (fifth-order polynomial) indicated that the first half of the sample data best fit a logarithmic curve whereas the second half best fit an exponential curve, which suggested that data were not parametric and random. To

(1501)	(1601)	(2000)	(2401)	(2402)	(2603)	(2604)	(2610)	(2701)	(2702)	(2801)	(2901)	(5301)
10.0 (1/10)	40.0 (4/10)	33.3 (5/15)	21.4 (3/14)	20.6 (7/34)	10.0 (1/10)	20.0 (1/5)	40.0 (4/10)	50.0 (1/2)	50.0 (1/2)	15.4 (4/26)	35.0 (7/20)	33.3 (4/12)
	18.8 (3/16)			25.0 (3/12)	28.6 (5/14)	30.0 (3/10)	20.0 (2/10)				13.3 (2/15)	5.3 (1/19)
					40.0 (4/10)	32.3 (11/34)	20.0 (4/20)					
					42.1 (16/38)	80.0 (25/30)	18.8 (3/16)					
					70.0 (21/30)	14.3 (1/7)	57.1 (4/7)					
					16.7 (2/12)	20.0 (2/10)						

FIG. 1. Positive batch data obtained per farm (henhouse numbers are given in the top row) for rank sum analysis. Data in each column are the percentage of spleens positive for serovar Enteritidis (number of positive spleens/number of spleens cultured). Each data set represents one batch.

analyze if different populations were represented in the sample data, rank summation was applied by stacking tied data points and maintaining rank place by following tied points with an appropriate number of places (Fig. 2b). Data from Fig. 1 that were less than 0.1% different were classified as ties. Curvolinear analysis (fifth-order polynomial) was used to detect standard curves, and three were detected in phase I data. From left to right in Fig. 2b, population I was part of a large area under a curve of negative or low-percentile data, population II formed a prominent standard curve that overlapped with population I, and population III was the smallest population and was composed of the highest-percentile data points. When the curves derived from rank analysis (Fig. 2a) and from rank sum analysis (Fig. 2b) were superimposed, it was seen that population III emerged as the sample data changed from a logarithmic to an exponential best curve fit (Fig. 2c).

Production of HMW LPS is associated with three serovar Enteritidis populations in experimentally infected chicks. To investigate commonalities between the field data collected from rodents and experimental data from chick models, chicks were infected with characterized lipopolysaccharide (LPS) variants to see if the distribution of data was parametric. This evaluation was especially important because there was a chance that the uneven sample sizes collected during the rodent survey could have introduced unacceptable variance that increased the chances of any one tie occurring. Thus the chick experiment was an opportunity to evaluate for the presence of multiple populations while controlling for sample size variance. For these experiments, chicks were housed 9 or 10 per isolator cage and infected intraperitoneally with 5×10^5 cells of one of five isogenic LPS variants that produce 0, 1, or 2 μg of rhamnose per μg of 2-keto-3-deoxyoctulosonic acid (KDO) as previously determined by compositional analysis (3, 4, 18). Spleens were assayed for numbers of bacteria as previously described (3, 18).

Of those chicks infected with variants with LPS O-antigen/core antigen (O/C) ratios of 2 in two trials, at least 500 CFU were recovered from 85% of the spleens, with means of 2,606 and 1,271 CFU per spleen recovered, and 100% of these spleens were positive for serovar Enteritidis. Of those chicks infected with avirulent variants with O/C ratios of 0 or 1, only 3.7% of spleens had at least 500 CFU, and 74% of these spleens were positive for serovar Enteritidis. Means were (i) 6.1 CFU for one isolate with an O/C ratio of 0 and (ii) 54 and 67 for two experiments conducted with an isolate with an O/C

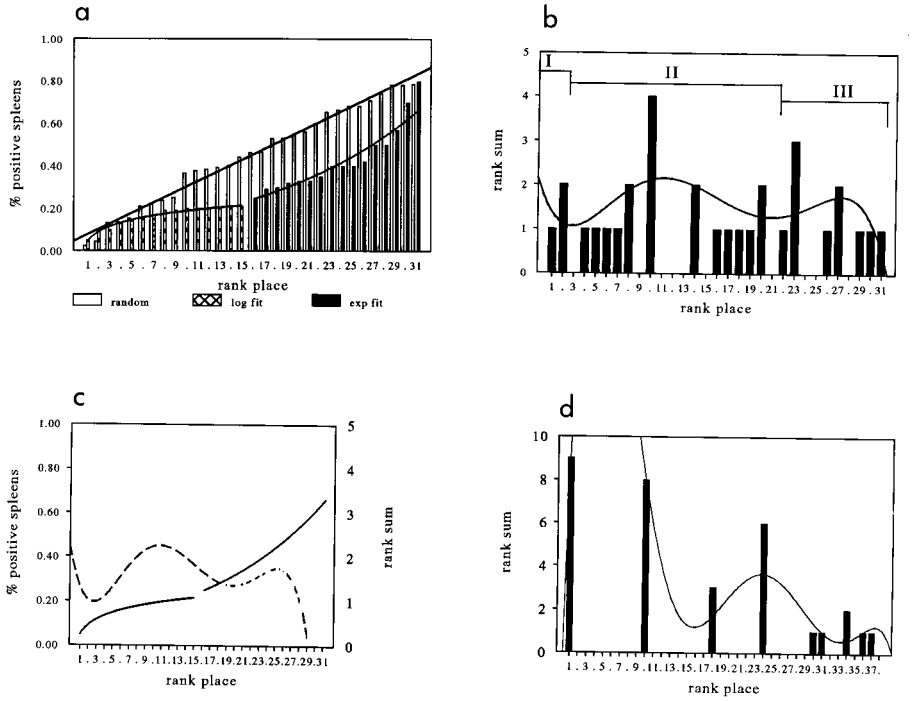

FIG. 2. Statistical analysis of spleens cultured positive for *S. enterica* serovar Enteritidis from naturally infected mice. (a) Rank analysis of positive batch data obtained from culturing the spleens of naturally infected mice. Data points (Fig. 1) were analyzed by ranking from least to greatest. Random numbers were generated by using Microsoft Excel statistical analysis software. (b) Rank summation and fifth-order polynomial analysis of mouse data. Data from Fig. 1 were rounded to 0.1 percentage point for the determination of ties. (c) Superimposition of rank and rank summation curves derived from mouse data. (d) Rank summation of data obtained from culturing the spleens of chicks after experimental challenge with LPS variants. Spleens positive for serovar Enteritidis but with fewer than two times the detection limit of 5 CFU per spleen for this assay (5 CFU) were called ties. All other data were called ties in increments of 100 CFU.

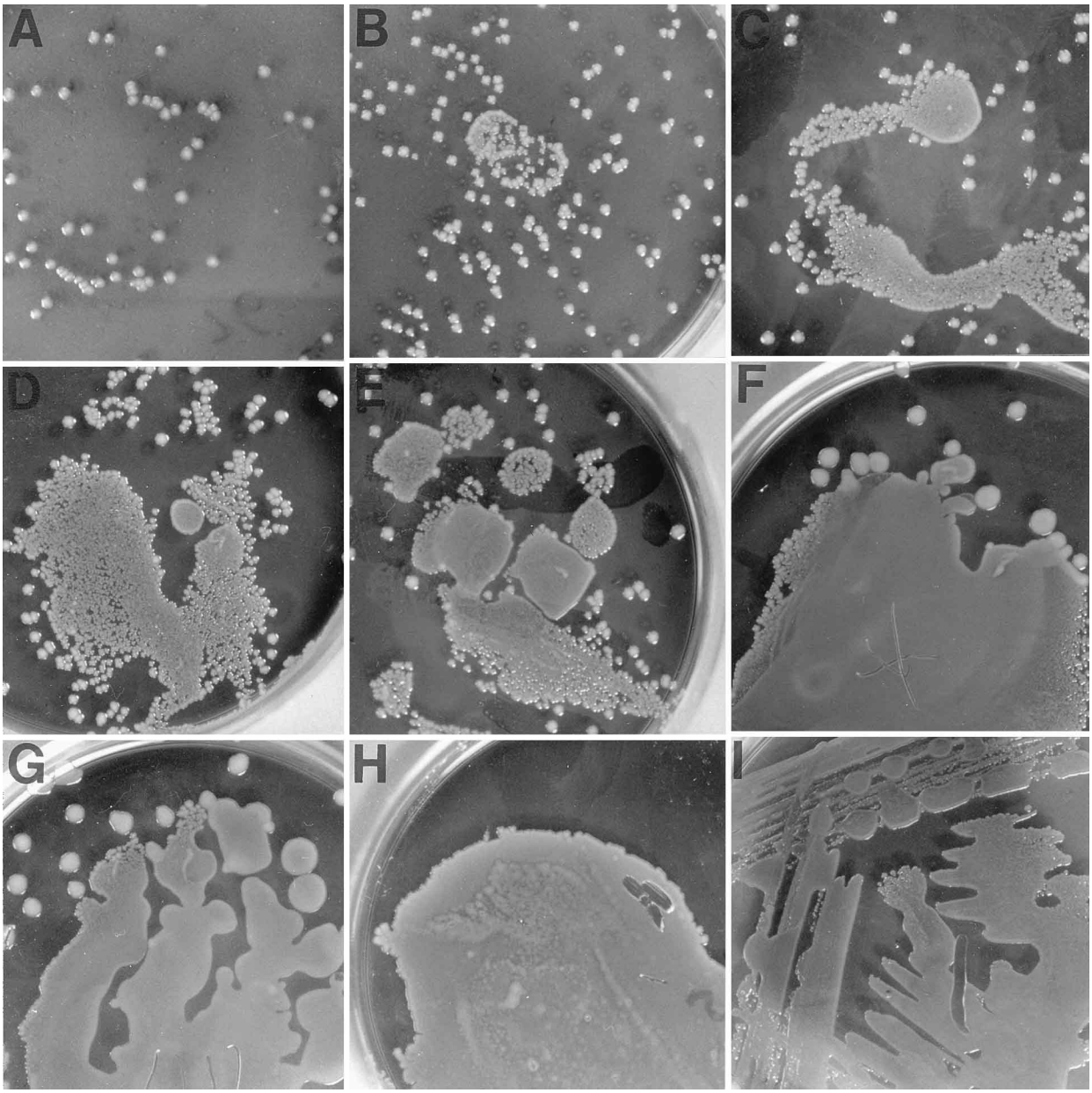

FIG. 3. Swarming migration of *S. enterica* serovar Enteritidis on 2% inhibitory brilliant green agar. (A) Spleen-invasive, non-egg-contaminating serovar Enteritidis on 2% brilliant green agar, passage 10; (B and C) migrating, spleen-invasive, egg-contaminating serovar Enteritidis, passages 1 and 2, respectively; (D to I) unpassaged migrating isolates as obtained from the spleens of naturally infected mice.

ratio of 1. Chick mortality within 3 days of challenge was 32% after challenge with serovar Enteritidis variants that had O/C ratios of 2 versus 7% for chicks challenged with variants with O/C ratios of 1. No deaths occurred in chicks infected with rough variants of serovar Enteritidis (O/C ratio of 0).

When rank summation was used to analyze all positive data points obtained from surviving chicks (raw data not shown), populations were nonparametric and similar to those identified by analysis of mouse data (Fig. 2d). Population III was composed exclusively of data obtained from chicks infected with serovar *Enteritidis* variants with O/C ratios of 2, whereas the other two populations were composed of data from all three LPS variants. There was a shift towards population 1 in the chick model using laboratory isolates in comparison to data obtained from culture of house mice (Fig. 2c and d).

HMW LPS is recoverable from spontaneously swarming mouse spleen isolates. Statistical analysis suggested that an emerging serovar Enteritidis population was associated with an exponential increase in the recovery of culture-positive spleens from mice. In chicks a similar population was associated with production of high-molecular-weight (HMW) LPS, as shown here and previously (3, 18). Further investigation of isolates producing HMW LPS indicated that they alone were capable of swarm migration across inhibitory agar (4). Swarming migration is important in microbial pathogenicity because it is associated with upregulation and hyperexpression of several

virulence factors (1). If such a correlation between swarming and production of HMW LPS exists for serovar Enteritidis, then spontaneously swarming isolates should yield HMW LPS.

Of 249 serovar Enteritidis isolates cultured from mouse spleens, 19 (7.6%) underwent spontaneous swarm migration on 2% brilliant green agar (Fig. 3), which was confirmed to be associated with hyperflagellation by scanning electron microscopy (data not shown). The LPS compositions of six of these swarming isolates were examined by using cells scraped from primary plates by published techniques (3, 18). Results indicated that spontaneously swarming isolates of serovar Enteritidis produced an unprecedentedly high O/C ratio, which averaged 3.1 μg (±0.41 μg) of rhamnose per μg of KDO for the six isolates analyzed (O/C ratio = 3.1). In comparison, previously characterized isogenic LPS variants yield O/C ratios of 1 for avirulent wild-type serovar Enteritidis isolates producing primarily low-molecular-weight (LMW) LPS and 2 for virulent variants producing 50% HMW LPS. LPS O/C ratios of 1, 2, and 3 represent average O-antigen repeating unit lengths of 5, 11, and 17 per core molecule, respectively, as determined from molar ratios assuming 2 KDO molecules per core molecule and assuming that 50% of core LPS is never linked to O antigen (5, 20). Since the maximum repeating unit length is 28 for a fraction of the LPS molecules recovered from serovar Enteritidis (6), an O/C ratio of 3.5 is probably near the theoretical limit of O-antigen production since it represents an average of 20 repeating units for every linked core molecule. O/C ratios higher than 3.5 have not yet been obtained, and all LPS samples analyzed to date contain at least a small fraction of LMW LPS (19). To assure that extracts were obtained from pure *S. enteritidis* cultures, plates covered with swarm colonies were further cultured to confirm that no other salmonellae or contaminants had been present on the initial culture plate.

Discussion. The results strongly indicate that the mouse is a carrier of invasive *S. enterica* serovar Enteritidis in the henhouse environment, which is an association that has been suggested by others (8). In addition, a statistical model for the dynamics of invasive serovar Enteritidis in animal populations was generated by analysis of the percentage of positive spleens from mice and by characterization of LPS of strains used to infect chicks. To date, no evidence conflicts with the proposal that the structure of LPS is a sensitive barometer of the ability of serovar Enteritidis to survive environmental stress, invade organs, and efficiently contaminate eggs (3, 4, 12, 13, 18, 19). Indeed, the models generated appear to fulfill Koch's postulates for causation of an infectious process, defined here as organ invasion and egg contamination. Understanding why strains differ in their ability to produce HMW LPS is perhaps central to understanding how serovar Enteritidis became the pathogen that it is today. Reasons for strain variation include mutation and the inevitable accumulation of allelic differences between bacterial populations that affect gene regulation (10, 14). Surface heterogeneity is part of the population biology of serovar Enteritidis associated with the ability of strains to contaminate eggs (3, 4, 19), whereas there has been no association made between virulence factor expression and the presence of stable mutation. There is evidence that laboratory media fail to support the growth of serovar Enteritidis populations that reach high cell densities (3.5×10^9 CFU/ml) while maintaining O/C ratios of at least 2 (4). Thus, inclusion of analysis of LPS structure in evaluation of serovar Enteritidis isolates recently obtained from outbreaks portends to provide useful epidemiological information that might aid in the control of this persistent pathogen.

Control measures must take into account that invasive serovar Enteritidis is detected for years in mouse populations. Bacterins are used as aids to prevent egg contamination, and results support the theory that vaccination is effective although failures occur. Live recombinant DNA vaccines are under consideration for use within the United States for laying hens, which might result in secondary exposure of mice. Not all secondary infections are harmless or beneficial, so the role that mice might have in propogating or harboring different pathotypes of serovar Enteritidis needs investigation. For monitoring serovar Enteritidis on farms, culturing of 30 mice monthly is suggested, although numbers less than 30 may provide useful information. No matter what type of vaccine is used, the incidence of serovar Enteritidis in mouse spleens might indicate waning immunity, the presence of virulent strains, and an increased potential for production of contaminated eggs.

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