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Comparison of the Prevalence of *Salmonella* Infection in Layer Hens from Commercial Layer Farms with High and Low Rodent Densities

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SUMMARY. A comparison on the prevalence of *Salmonella* infection in layer hens from commercial layer farms with high and low rodent densities was investigated. Out of 280 laying hens sampled from three commercial layer farms with high rodent densities, *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) was isolated from 20 (7.14%) hens and *Salmonella enterica* subsp. *enterica* serovar Infantis (*Salmonella* Infantis) from three (1.07%) hens. In contrast, layer hens sampled from four commercial layer farms with low rodent densities were negative for any salmonellae. Significant differences ($P < 0.05$) in the isolation rates of *Salmonella* from various organs of infected layer hens were also noted. For *Salmonella* Enteritidis, liver (55.0%) and the oviduct (55.0%) had the highest isolation rates while all *Salmonella* Infantis isolates were from the oviduct. Pulsed field gel electrophoresis (PFGE) analysis of *Bln*I-digested chromosomal DNA of *Salmonella* Enteritidis isolated from layer hens and rodents showed similar patterns. PFGE analysis of *Salmonella* Infantis isolated from layer hens, rodents, eggs, and the environment yielded identical patterns. In this study, the significantly higher prevalence rate ($P < 0.05$) of *Salmonella* Enteritidis and *Salmonella* Infantis in layer hens from high rodent density farms could be attributed to the high rodent population density. The persistent *Salmonella* Enteritidis and *Salmonella* Infantis infection inside layer houses may have been amplified by the increasing numbers in the rodent population over the years, which increased the opportunity for environment-rodent-chicken interaction and the transmission of salmonellae to chickens. Monitoring of salmonellae from rodents inside poultry premises is recommended to be an effective additional tool in the assessment of the *Salmonella* status of layer flocks.

RESUMEN. Comparación de la prevalencia de la infección por *Salmonella* en gallinas de postura de granjas comerciales con densidades de roedores altas o bajas.

Se realizó una comparación de la prevalencia de la infección por *Salmonella* en gallinas de postura en granjas comerciales con densidades de roedores alta o baja. De 280 gallinas de postura que fueron muestreadas en tres granjas comerciales con una densidad alta de roedores, la *Salmonella enterica* subsp. *enterica* serovariedad Enteritidis (*Salmonella* Enteritidis), se aisló de 20 gallinas (7.14%) y la *Salmonella enterica* subsp. *enterica* serovariedad Infantis (*Salmonella* Infantis) se aisló de tres gallinas (1.07%). Por el contrario, las gallinas de postura muestreadas de cuatro granjas comerciales con una densidad baja de roedores fueron negativas para el aislamiento de cualquier salmonela. Se observaron también diferencias significativas ($P < 0.05$) en las tasas de aislamiento de *Salmonella* a partir de los diversos órganos de gallinas de postura infectadas. Para el caso de *Salmonella* Enteritidis, el hígado (55.0%) y el oviducto (55.0%) mostraron las tasas más altas de aislamiento, mientras que todos los aislamientos de *Salmonella* Infantis fueron del oviducto. El análisis mediante la electroforesis en gel con campo de pulsaciones (PFGE) del ADN cromosómico digerido con la enzima *Bln*I obtenido de los aislamientos de *Salmonella* Enteritidis de gallinas de postura y de roedores mostraron patrones similares. El análisis por PFGE de *Salmonella* Infantis aislado de gallinas de postura, de roedores, huevos, y del medio ambiente produjo patrones idénticos. En este estudio, la tasa de prevalencia significativamente mayor ($P < 0.05$) de la *Salmonella* Enteritidis y de *Salmonella* Infantis en gallinas ponedoras de las granjas con alta densidad de roedores podría atribuirse a la alta densidad de población de roedores. La persistencia de la infección por *Salmonella* Enteritidis y por *Salmonella* Infantis dentro de las casetas de aves de postura puede haber sido amplificada por el aumento en la población de roedores en los últimos años, lo que aumentó la posibilidad de interacción entre el medio ambiente, los roedores y el pollo y la transmisión de *Salmonella* a los pollos. La vigilancia de la *Salmonella* de los roedores dentro de las instalaciones de aves de postura se considera como un instrumento adicional efectivo en la evaluación del estado sanitario de las gallinas postura.

Key words: commercial layer farm, pulsed field gel electrophoresis, *Salmonella* Enteritidis, *Salmonella* Infantis, rodents

Abbreviations: DHL = desoxycholate hydrogen sulfide lactose; HTT = Hajna tetrathionate; PFGE = pulsed field gel electrophoresis; RI = rodent index; *Salmonella* Enteritidis = *Salmonella enterica* subsp. *enterica* serovar Enteritidis; *Salmonella* Infantis = *Salmonella enterica* subsp. *enterica* serovar Infantis

There are over 2,000 *Salmonella* serovars presently recognized. Some *Salmonella* serovars, such as *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis), *Salmonella* Typhimurium, *Salmonella* Infantis, and *Salmonella* Newport are just some of the serotypes that cause diarrheal diseases and pose a public health threat to humans. Most of the infections caused by these serovars can be traced back to dairy, poultry, and meat products. In particular, chicken meat and egg products are considered a *Salmonella* high-risk food.

In Japan, *Salmonella* Enteritidis and *Salmonella* Infantis are the top two serovars associated with human salmonellosis cases during the past 4 yr (8). Egg contents may become contaminated with *Salmonella* from soiling with the feces of infected chickens (1). There are also some evidences that *Salmonella* Enteritidis organisms gain access to egg contents by migrating from the cloaca to the reproductive organs. However, recent studies have shown that *Salmonella* Enteritidis contaminates the egg via transovarian infection following systemic infection and localizing in the ovaries

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and oviducts (6,9,13,16). *Salmonella* Enteritidis migrate inside the yolk before shell deposition. This unique ability of *Salmonella* Enteritidis to contaminate the egg contents routinely is a major public health concern to human consumers.

Salmonella infections in layer flocks are usually mild or subclinical with little or no effect on egg production (7,11). Consequently, infected flocks are difficult to identify (14). Hence, assessment of the status of *Salmonella* contamination in layer farms is usually based on isolation of *Salmonella* from environmental and egg samples. Monitoring for salmonellae from these samples provides an indirect epidemiologic link between *Salmonella* and the infected hens. Although there are many reports about *Salmonella* isolation from experimentally inoculated hens, there are limited studies regarding the prevalence of *Salmonella* Enteritidis in naturally infected laying hens (3,14,15). Moreover, several researchers have suggested that rodents may be important vectors and amplifiers of *Salmonella* infection in layer farms (2,5). Hence, it is therefore interesting to determine and compare the prevalence of *Salmonella* infection in layer hens housed in layer premises with high and low rodent densities and to elucidate the possible epidemiologic link between rodents and layer hens.

MATERIALS AND METHODS

Poultry farms. Seven commercial layer farms with windowless, multiple house-in-line complexes, which consist of several hen houses that are connected by conveyor belts that transport eggs to the egg processing facility for grading and packing, were used in this study. Three of the seven farms (Farms A, B, and C) were confirmed to have high rodent density scores while four of the seven farms (Farms D, E, F, and G) were considered to have low rodent density scores based on visual inspection and trapping. All of the farms were environmentally controlled and were operated with automated systems.

Estimation of rodent population. Visual inspections for the presence of rodents, such as presence of rodent feces, rodent burrows, and the rodent itself, were performed during the daytime and at night. Rodent density was estimated using a rodent index (RI); the RI is based on the total number of rodents caught in a designated number of traps over a specific period of time (5). Calculations to estimate RI are summarized in Table 1.

Rodent trapping. Rodent trapping was performed as previously described (10). In brief, rodents were trapped using adhesive traps and pipe traps (custom-made traps by poultry workers). Traps were baited with chicken feed and various kinds of grains and seeds and were placed where rodents regularly traveled. Traps were checked every 24–48 hr. Thirty-three live rodents were caught by pipe traps and a total of 818 rodents were obtained from adhesive traps. All live rodents were killed by chloroform inhalation. All rodents caught were placed individually in a plastic bag on ice, stored at 4 C in the laboratory, and cultured within 1–3 days after trapping. In total, 851 rodents were examined wherein all were identified as roof rats *(Rattus rattus).*

Isolation of *Salmonella*. A total of 380 laying hens from the seven different farms were randomly sampled for *Salmonella* isolation. Chickens were killed by cervical dislocation. Each chicken was disinfected with a 3:1 solution of 70% ethyl alcohol and 10% iodine. The abdominal cavity was opened aseptically. An approximately 1 to 2-g portion of the spleen, liver, kidney, ovary, oviduct, cloaca, and ceca were put into a sterile plastic bag containing 50 ml of heart infusion broth (Eiken, Tokyo, Japan) and then incubated for 48 hr at 42 C. One milliliter of this culture was then inoculated to 9 ml of Hajna tetrathionate (HTT) broth (Eiken) and incubated for 18 hr at 37 C. A loop-full from the HTT culture was then streaked onto a desoxycholate hydrogen sulfide lactose (DHL) agar (Eiken) and incubated for 18 hr at 37 C. *Salmonella*-suspect colonies were confirmed and identified by biochemical tests and by serotyping with *Salmonella* O and H antigens (Denkaseiken, Tokyo, Japan). The isolation and characterization of *Salmonella* from roof rats, eggs, and environmental samples were done in an earlier study involving the same layer flocks and had previously been first reported by the authors (10).

Pulsed field gel electrophoresis (PFGE) analysis. *Bln*I-digested chromosomal DNA of *Salmonella* isolates obtained from organs of laying hens, rodents, eggs, and environmental samples were analyzed by PFGE. A total of 20 *Salmonella* Enteritidis and three *Salmonella* Infantis strains isolated from layer hens, and several *Salmonella* Enteritidis and *Salmonella* Infantis strains isolated from rats, eggs, and environmental samples, were characterized by PFGE analysis. *Salmonella* chromosomal DNA for PFGE analysis was prepared as previously described (12). Chromosomal DNA in each plug was digested with 20 U of *Bln*I (Takara, Kyoto, Japan) at 37 C for 18 hr. PFGE was performed using a CHEF-DR III apparatus (Bio-Rad, Tokyo, Japan) in gels of 1% agarose (Bio-Rad) on 0.5× Tris-borate EDTA buffer (Bio-Rad) for 21 hr at 200 volts and 14 C with a pulse time ranging from 2 to 43.2 sec. The gels were stained with ethidium bromide (Bio-Rad) and photographed with an ultraviolet illuminator (Atto Systems, Osaka, Japan). A DNA lambda ladder was used as a molecular marker (Bio-Rad). DNA fragments were analyzed visually and roman letters were used for assigning the different pulsed-field patterns generated.

Statistical analyses. A test on two independent proportions was used in comparing the level of infection of *Salmonella* between the high rodent density farms (Farms A, B, and C) and the low rodent density farms (Farms D, E, F, and G). A test between two nonindependent proportions in nonoverlapping and overlapping classes was performed in comparing the isolation rate of *Salmonella* Enteritidis and *Salmonella* Infantis from the different organs of layer hens.

RESULTS

Salmonella Enteritidis were isolated from 20 (5.2%) out of 380 laying hens. All of the *Salmonella* Enteritidis-infected hens were from farms (A, B, and C) that were highly infested with *Salmonella* Enteritidis-positive rats. *Salmonella* Infantis were isolated in three (0.8%) layer hens from Farm A only (Table 2). There were no *Salmonella* spp. detected from laying hens housed in Farms D, E, F, and G.

Table 1. Estimation of rodent density of each farm.

Farm	Visual inspection	Rodent index[A]	Estimated rodent density
A	Numerous sightings during daytime and at night	47	High
B	Numerous sightings during daytime and at night	33	High
C	Numerous sightings during daytime and at night	29	High
D	No rodent sightings	0	Low
E	Occasional sightings at night	3	Low
F	No rodent sightings	0	Low
G	No rodent sightings	0	Low

[A]Adapted from Henzler and Opitz (5). Rodent index (RI) = [(number of rodents caught on all traps/number of functioning traps) × total number of traps/number of days traps were set] × 7. RI = 1–10 (low density); RI = 11– 25 (moderate density); RI > 25 (high density).

Table 2. Isolation of *Salmonella* spp. from different organs of layer hens.

Farm	No. of hens examined	*Salmonella* spp.	No. of positive (%)	Liver	Spleen	Kidney	Intestine	Ovary	Oviduct	Cloaca
A	140	*Salmonella* Enteritidis	8 (5.7)*	4	3	0	0	1	5	0
		Salmonella Infantis	3 (2.1)*	0	0	0	0	0	3	0
B	60	*Salmonella* Enteritidis	5 (8.3)*	2	2	0	0	0	2	2
C	80	*Salmonella* Enteritidis	7 (8.8)*	5	3	0	1	1	4	0
D	20	*Salmonella* spp.	0 (0)	0	0	0	0	0	0	0
E	40	*Salmonella* spp.	0 (0)	0	0	0	0	0	0	0
F	20	*Salmonella* spp.	0 (0)	0	0	0	0	0	0	0
G	20	*Salmonella* spp.	0 (0)	0	0	0	0	0	0	0
Total	380	*Salmonella* Enteritidis (%)	20 (5.2)	11 (55.0)a	8 (40.0)a	0 (0)b	1 (5.0)b	2 (10.0)b	11 (55.0)a	2 (10.0)b
		Salmonella Infantis (%)	3 (0.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (100)a	0 (0)

*Comparison between Farms A, B, and C *vs.* Farms D, E, and F is significantly different at $\alpha = 0.05$ (Zc 1.915, *P*-value 0.0277). In each row, means with different lowercase letters are significantly different at $\alpha = 0.01$ ($P < 0.05$). *P*-values: liver *vs.* spleen, 0.2156000; liver *vs.* kidney, 0.0000002; liver *vs.* intestine, 0.0000022; liver *vs.* ovary, 0.0000177; liver *vs.* oviduct, 0.5000000; liver *vs.* cloaca, 0.0000177; spleen *vs.* kidney, 0.0001007; spleen *vs.* intestine, 0.0004355; spleen *vs.* ovary, 0.0015320; spleen *vs.* oviduct, 0.2156000; spleen *vs.* cloaca, 0.0175000; kidney *vs.* intestine, 0.1521000; kidney *vs.* ovary, 0.0674000; kidney *vs.* oviduct, 0.0000002; kidney *vs.* cloaca, 0.0674000; intestine *vs.* ovary, 0.1521000; intestine *vs.* oviduct, 0.0000022; intestine *vs.* cloaca, 0.2801000; ovary *vs.* oviduct, 0.0000177; ovary *vs.* cloaca, 0.5000000; oviduct *vs.* cloaca, 0.0002627.

Isolation rates of *Salmonella* from various organs of laying hens were investigated. The oviduct (55.0%) and liver (55.0%) of infected layer hens were the organs with the highest isolation rates ($P < 0.05$) for *Salmonella* Enteritidis. In contrast, only two (10%) cecal samples were positive for *Salmonella* Enteritidis. Interestingly, three oviduct samples from Farm A were also positive for *Salmonella* Infantis (Table 2).

Several *Salmonella* Enteritidis and *Salmonella* Infantis strains isolated from layer hens and roof rats from different farms were characterized by PFGE analysis to elucidate the possible epidemiologic link between roof rats and layer hens. Complete results on the isolation of *Salmonella* from roof rats, eggs, and environmental samples from these layer flocks were previously reported by the

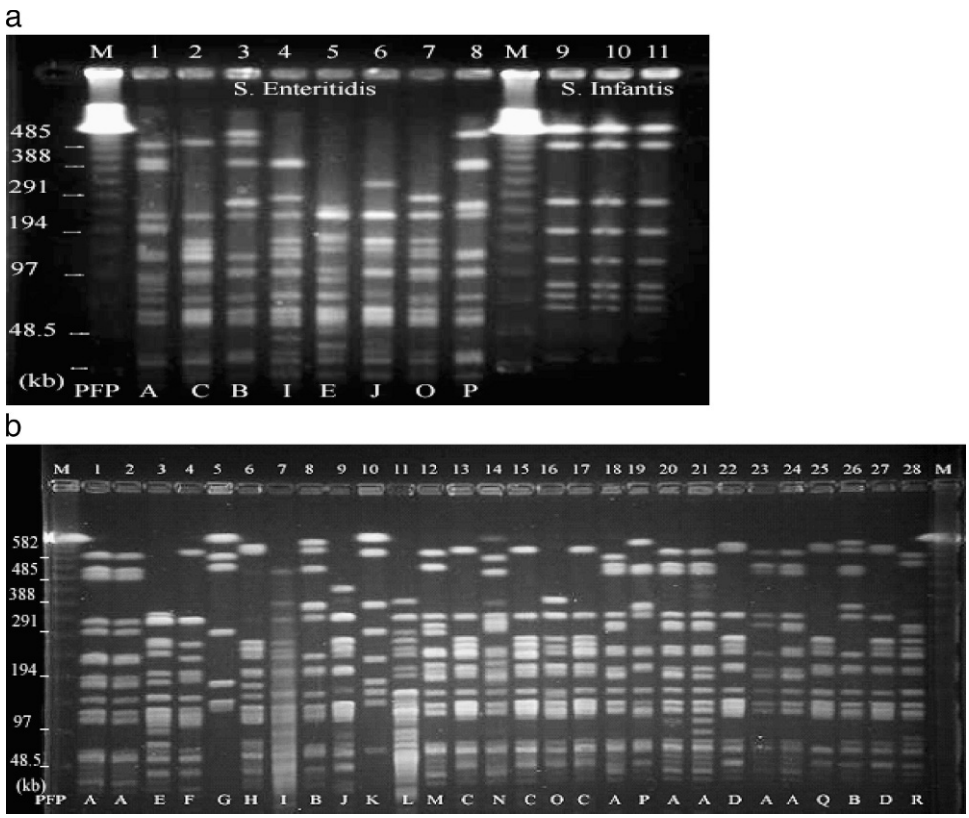

Fig. 1. Comparison of PFGE patterns of *Bln*I-digested chromosomal DNA of *Salmonella* Enteritidis isolates from layer chickens, roof rats, eggs, and environmental samples of Farm A. (a) Lanes 1–8: *Salmonella* Enteritidis isolates from layer chickens. Lanes 9–11: *Salmonella* Infantis isolates from layer chickens. (b) Lanes 1–13: *Salmonella* Enteritidis isolates from roof rats; six PFGE patterns were similar with PFGE patterns of *Salmonella* Enteritidis isolates from layer chickens. Lanes 14–23: *Salmonella* Enteritidis isolates from egg samples; two PFGE patterns were similar with PFGE patterns of *Salmonella* Enteritidis isolates from roof rats. Lanes 24–28: *Salmonella* Enteritidis isolates from environmental samples; two PFGE patterns were similar with PFGE patterns of *Salmonella* Enteritidis isolates from roof rats. M = molecular marker (DNA lambda ladder). Letters at the bottom of each figure indicate the assigned pulsed-field patterns generated.

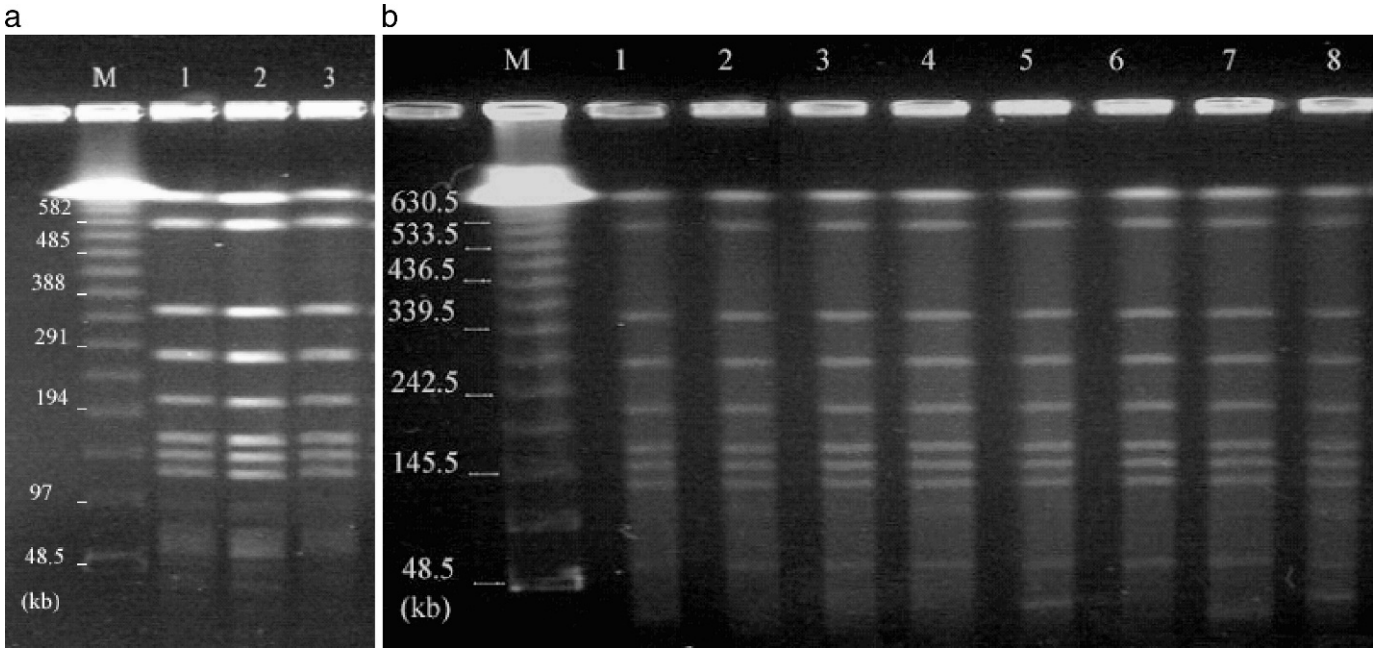

Fig. 2. Comparison of PFGE patterns of *Bln*I-digested chromosomal DNA of *Salmonella* Infantis isolates from layer chickens, roof rats, eggs, and environmental samples of Farm A. All *Salmonella* Infantis isolates showed similar PFGE patterns. (a) Lanes 1–3: *Salmonella* Infantis isolates from layer chickens. (b) Lanes 1–2: *Salmonella* Infantis isolates from egg samples. Lanes 3–5: *Salmonella* Infantis isolates from environmental samples. Lanes 6–8: *Salmonella* Infantis isolates from roof rats. M = molecular marker (DNA lambda ladder).

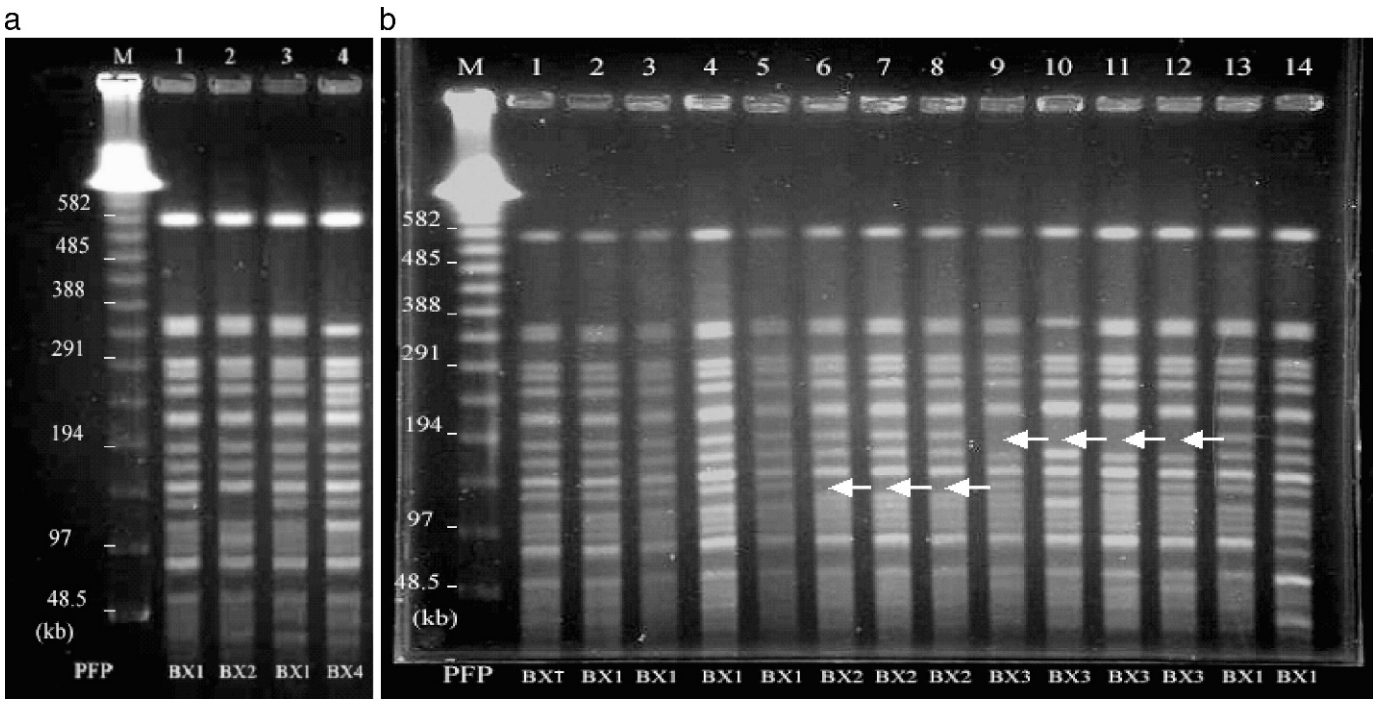

Fig. 3. Comparison of PFGE patterns of *Bln*I-digested chromosomal DNA of *Salmonella* Enteritidis isolates from layer chickens, roof rats, eggs, and environmental samples of Farm B. (a) Lanes 1–4: *Salmonella* Enteritidis isolates from layer chickens. (b) Lanes 1–4: *Salmonella* Enteritidis isolates from environmental samples; one PFGE pattern was similar with PFGE patterns of *Salmonella* Enteritidis isolates from roof rats. Lanes 5–9: *Salmonella* Enteritidis isolates from egg samples; two PFGE patterns were similar with PFGE patterns of *Salmonella* Enteritidis isolates from roof rats. Lanes 10–14: *Salmonella* Enteritidis isolates from roof rat samples; one PFGE pattern was similar with PFGE patterns of *Salmonella* Enteritidis isolates from layer chickens. M = molecular marker (DNA lambda ladder). Letters at the bottom of the figure indicate the assigned pulsed-field patterns generated. Arrows indicate the missing DNA fragments.

authors (10). *Bln*I-digested chromosomal DNA of *Salmonella* Enteritidis from laying hens from Farm A yielded 7 distinct pulsed-field patterns (Fig. 1a). Some of the *Salmonella* Enteritidis isolates from layer hens, roof rats, eggs, and the environment shared similar patterns (A, B, C, E, I, O, and P; Fig. 1a,b). In addition, *Bln*I-digested chromosomal DNA of *Salmonella* Infantis isolated from hens, roof rats, eggs, and the environment all yielded an identical pattern (Fig. 2a,b). In Farm B, *Salmonella* Enteritidis strains generated very closely related pulsed-field patterns (PFP BX1, BX2, BX3, BX4; Fig. 3a,b). For Farm C, *Bln*I-digested chromosomal DNA of *Salmonella* Enteritidis isolates from layer hens, roof rats, eggs, and the environment also shared an indistinguishable pulsed-field pattern (Fig. 4). However, pulsed-field patterns of *Salmonella* Enteritidis isolates from each farm were distinct from each other (Table 3).

DISCUSSION

Although most of the *Salmonella* serovars, including *Salmonella* Enteritidis and *Salmonella* Infantis, are not actually serious pathogens in chickens, they are a major public health concern, globally, due to their incrimination in human salmonellosis outbreaks. In particular, *Salmonella* Enteritidis accounted for more than 25% of human salmonellosis outbreaks in Japan (8).

Although there are high incidences of food poisoning cases caused by *Salmonella* Enteritidis in Japan, there are limited published reports about the prevalence of natural *Salmonella* Enteritidis infections in layer hens in the field, the primary source of the contaminated eggs. Assessments of the *Salmonella* contamination status of layer flocks are usually based on isolations of *Salmonella* organisms from the farm environment such as house dust, chicken manure, and egg samples. However, these are indirect assessments of the *Salmonella* status of the laying flocks.

Ebel *et al*. (3) reported that only 3% (or 607) out of 23,431 of the spent hens examined in the United States were positive for *Salmonella* Enteritidis. In Canada, Poppe *et al*. (14) have also reported that 4.5% (26/580) of the hens necropsied were positive for *Salmonella* Enteritidis. Sunagawa *et al*. (15) have isolated *Salmonella* Enteritidis from only 0.4% (or 3) of 740 spent hens in Hokkaido, Japan, and all of these chickens were housed in windowless premises. In the present study, 5.3% (or 20) of the 380 laying hens were infected by *Salmonella* Enteritidis, and these results are comparable to the data reported by Poppe *et al*. (14) in Canada. Results of isolation of *Salmonella* from individual organs showed that for *Salmonella* Enteritidis, the oviduct (55%) and the liver (55%) had the significantly highest isolation rates ($P < 0.05$) followed by the spleen (40%). These findings suggest that *Salmonella* Enteritidis infection in naturally infected hens is systemic, a necessity for *Salmonella* Enteritidis organisms to invade the reproductive organs and contaminate the inside of the eggs. In contrast, isolation rates of *Salmonella* Enteritidis from ceca (5%) and cloacal (10%) samples were low. These lower isolation rates of *Salmonella* Enteritidis from cecal and cloacal samples in this study are probably due to the low excretion rates of *Salmonella* Enteritidis from systemic organs into the intestinal tract, its diminished ability to persist in the intestinal tract following systemic or oral infection routes, or both. Similar findings were noted by Gast *et al*. (4) following experimental inoculation of *Salmonella* Enteritidis in layer chickens after prior serial passage *in vivo*.

In this study, it is interesting to note that all of the chickens that were positive for *Salmonella* Enteritidis were housed in poultry premises (Farms A, B, and C) which were heavily infested by roof rats. Coincidentally, these roof rats were also infected by *Salmonella* Enteritidis (10). In addition, environmental samples and eggs from Farms A, B, and C were also contaminated by *Salmonella* Enteritidis (10). In contrast, no *Salmonella* spp. were detected in layer hens originating from Farms D, E, F, and G where rodent densities were low.

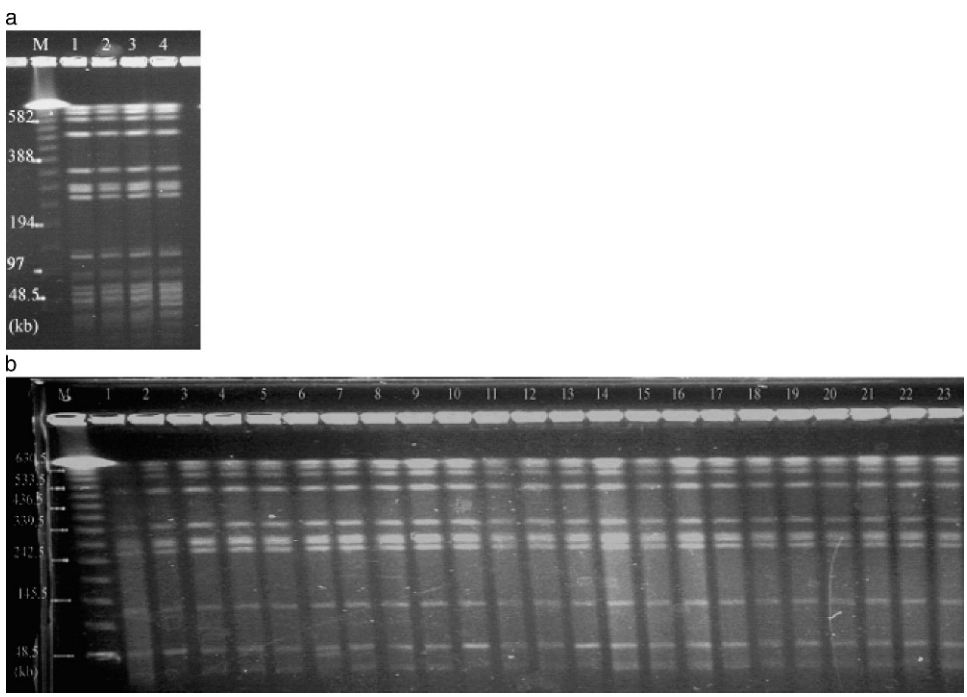

Fig. 4. Comparison of PFGE patterns of *Bln*I-digested chromosomal DNA of *Salmonella* Enteritidis isolates from layer chickens, roof rats, eggs, and environmental samples of Farm C. All *Salmonella* Enteritidis isolates showed similar PFGE patterns. (a) Lanes 1–4: *Salmonella* Enteritidis isolates from layer chickens. M = molecular marker (DNA lambda ladder). (b) Lanes 1–7: *Salmonella* Enteritidis isolates from environment samples. Lanes 8–14: *Salmonella* Enteritidis isolates from egg samples. Lanes 15–23: *Salmonella* Enteritidis isolates from roof rat samples. M = molecular marker (DNA lambda ladder).

Table 3. Summary of pulsed-field patterns generated by *Salmonella* Enteritidis strains isolated from layer hens.

Source of chickens	Number of isolates	PFGE patterns generated
Farm A	8	A (1)
		B (1)
		C (1)
		E (1)
		I (1)
		J (1)
		O (1)
		P (1)
Farm B	5	BX1 (3)
		BX2 (1)
		BX4 (1)
Farm C	7	No letter (7)

By PFGE analysis, the epidemiologic link of *Salmonella* Enteritidis infection between layer hens and roof rats inside the poultry houses was elucidated. Similar PFGE patterns (A, B, C, E, I, and J) were shared by *Salmonella* Enteritidis from roof rats and layer hens; this indicates these strains are very closely related to each other. The persistent *Salmonella* Enteritidis infection inside the layer houses may have been amplified by the increasing numbers in the rodent population over the years and, thereby, an increasing opportunity for environment-rat-chicken interaction. Even though replacement pullets were obtained from *Salmonella*-free breeders and rearing units, it is very plausible that they can be infected by *Salmonella* Enteritidis via rodents; this is probably one of the major reasons why layer houses can be persistently infected by *Salmonella* Enteritidis even when the facilities were thoroughly cleaned and disinfected. It is, therefore, noteworthy to suggest that the practice of monitoring of *Salmonella* Enteritidis in rodents inside the poultry premises may be used as an additional, effective tool in the assessment of the *Salmonella* status of layer flocks.

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