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Salmonella colonization in commercial pet turtles (*Pseudemys scripta elegans*)

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SUMMARY

An epidemiological survey was conducted on two commercial turtle farms in southern Louisiana to determine the reason for an apparent increase in the prevalence of *Salmonella* spp. in turtle hatchlings at the time of pre-export certification examination.

Pond water was consistently found to be contaminated (6/36 samples) with either *Salmonella newport*, *S. arizonae*, or *S. poona*. Environmental specimens obtained from eggs and turtle hatcheries (204 specimens) failed to yield *Salmonella* spp. A sample comprising 197 hatchlings, derived from a batch previously demonstrated to be contaminated, showed a salmonella prevalence of 12%, with *S. arizonae* and *S. poona* the only serotypes isolated. Four serotypes of *Salmonella* sp. isolated by a certifying laboratory in 1988, and 20 salmonella isolates obtained from hatchling turtles, were all resistant to gentamicin. The emergence of gentamicin resistance in *Salmonella* spp. isolated from turtles will reduce the effectiveness of preventive measures in use in Louisiana since 1984.

INTRODUCTION

The role of pet turtles as a source of salmonellosis in children was documented in 1965 [1]. Subsequent epidemiological studies showed that 23% of families in which a diagnosis of salmonellosis had been made owned or had kept a turtle, compared with only 6% of 248 controls [2]. Based on an annual sale of 15 million hatchlings, it was estimated that approximately 4% of all United States households maintained one or more turtles, each of which had an average lifespan of only 2 months [3]. This report evaluated five United States surveys which showed estimates of turtle-related salmonellosis ranging from 11% of all cases of juvenile salmonellosis in Georgia to 22% in Connecticut. In 1972, following the lead of Washington state, the United States Food and Drug Administration required certification of freedom from salmonella infection prior to interstate shipment of consignments of hatchling turtles. Subsequently 38% of all batches of turtles certified free of *Salmonella* spp. were shown to be contaminated, and therefore a total ban on interstate shipment of hatchlings in the United States was imposed in 1974. This action resulted in a marked decline in salmonellosis in children aged 1–9 years, as shown by a retrospective survey extending from 1971

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through 1975 [4]. A significant outbreak of *Salmonella pomona* infection occurred in Puerto Rico in 1984 following illegal shipment of turtles from Louisiana. It was calculated that 15% of all infant salmonellosis could be attributed to turtles in the year following importation, and that a diagnosed case resulted from each 1000 turtles sold [5]. *Salmonella pomona* was also identified in batches of turtles shipped from Louisiana to Guam and Yugoslavia [6]. It is generally accepted that immature turtles, 'which serve as a biological sponge for salmonella', [7] are inappropriate pets for children [8].

Epidemiological studies have been carried out on turtle farms in Louisiana [9, 10]. Salmonella was consistently isolated from pond water and soil samples in the vicinity of nests. In addition, turtle hatchlings were shown to excrete *Salmonella* spp. A 2-year longitudinal study conducted on a commercial turtle farm in Louisiana failed to show any evidence of transovarial infection. It was concluded that eggs were infected at the time of oviposition when bursal fluid, derived from pond water, is ejected over the clutch [11]. In an attempt to reduce the vertical transmission of Salmonella infection, eggs were immersed in solutions of either oxytetracycline or chloramphenicol at antibiotic concentrations ranging from 1000–2000 μg/ml [10]. Treatment was associated with reduced excretion of *Salmonella* spp. by hatchlings, and was a relatively efficient method of decontamination, providing eggs were free of organic matter at the time of decontamination and that antibiotic solutions were maintained at bactericidal levels. A refinement of this technique was introduced in 1983 when gentamicin was used to treat 48-h-old eggs using equipment to permit immersion in the antibiotic under negative pressure [13]. A 40% *Salmonella* spp. recovery rate was obtained from turtle hatchlings derived from eggs not immersed in an antibiotic solution [14]. In contrast, immersion of eggs in 500 μg/ml gentamicin sulphate solution using pressure differential equipment lowered the infection rate in hatchlings to 0·15%. Recommendations concerning decontamination, and procedures to immerse turtle eggs in gentamicin, were incorporated into official rules issued by the Louisiana Department of Agriculture and Forestry in 1985 issued pursuant to the Diseases of Animals Act. The Louisiana state regulations require that a random sample comprising 60 turtles from each batch should be submitted and examined for the presence of salmonella infection before export certificates are issued. Each sample to be certified is divided into 12 replicates of 5 turtles each; these are killed by exposure to chloroform and then homogenized (12) and examined for *Salmonella* spp. by an approved laboratory using a prescribed enrichment and isolation technique (15).

During 1988, 5 out of 115 submissions from 28 farms yielded *Salmonella* spp. at the time of examination for export certification. This fact, together with reports from importing countries of the presence of salmonella in batches of turtles previously certified as free of this organism prompted a review of current methods of hygiene and egg-treatment. In addition, the statistical validity of the sampling protocol as specified by relevant legislation was evaluated.

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MATERIALS AND METHODS

Sample collection

Water, soil, turtle eggs, and cloacal swabs from adult turtles from two farms (designated A and B) in south Louisiana were examined for *Salmonella* spp. Water samples from ponds and hatcheries were collected in sterile Whirl Pak® Thio-Bags™ (Nasco, Inc., Fort Atkinson, Wis., USA); soil and eggs were collected in plain sterile Whirl-Pak® bags (Nasco). Culturette® swabs (Baxter Healthcare Corporation, McGaw Park, Ill, USA) containing liquid Modified Stuart's Bacterial Transport Medium were used to obtain cloacal swabs from adult turtles shortly after oviposition.

Specimens were transported directly to the laboratory where procedures to isolate *Salmonella* spp. were performed. Samples were held under refrigeration (4 °C) until processed. All cultures were initiated within 24 h of collection.

Bacteriology

All samples were enriched in selenite broth (Difco Laboratories, Detroit, Mich., USA) prior to plating onto differential solid media.

Each bag containing water was thoroughly mixed by vigorous shaking for 30 seconds. A 2 ml amount was transferred by sterile pipette to 10 ml selenite broth. Water from beakers containing hatchling turtles was agitated, and 2 ml inoculated into selenite broth. Tubes were incubated overnight at 35 °C.

Bags containing soil were pressed to break up aggregates, then shaken for 30 seconds. A sterile cotton swab moistened with selenite broth was inserted into the mixed soil to obtain a representative specimen and then placed into a tube containing selenite broth and incubated as described above.

Both the shell and contents of freshly-laid turtle eggs were examined for *Salmonella* spp. A sterile cotton swab moistened with selenite broth was rubbed over the surface of the shell, then placed into selenite broth and incubated. After sampling the exterior, soil debris on the surface was removed by wiping; the shell was rinsed with ethanol and allowed to dry. A 0·5 cm incision was made in the shell using a sterile scalpel. A sterile swab was inserted through the aperture in the shell to mix and obtain a representative sample of the contents. The swab was then transferred to selenite broth.

Culturette® swabs were placed directly into selenite broth and incubated.

Isolation and identification

Hektoen enteric agar (Difco) and brilliant green agar (Difco) plates were inoculated from the selenite broth. After overnight incubation at 35 °C, plates were inspected for colonies typical of *Salmonella* spp. Oxidase-negative, non-lactose-fermenting colonies were tested against polyvalent salmonella O antiserum for serogroups A–I and Vi (Difco). Colonies causing agglutination of the antiserum were transferred to tubes of Triple Sugar Iron agar (TSI) (Difco) and Lysine Iron Agar (LIA) (Difco) for initial screening. Because some *Salmonella arizonae* strains do not react with polyvalent antiserum, non-agglutinating colonies with the appearance of salmonella were also inoculated into TSI and LIA.

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Media were incubated overnight at 35 °C, then examined. Lactose non-fermenters which produced H_2S and showed a positive lysine decarboxylase reaction were inoculated into the Minitek™ Enterobacteriaceae II Differentiation System (BBL Microbiology Systems, Cockeysville, Md., USA). Organisms which were identified as *Salmonella* sp. were sent to the United States Department of Agriculture Animal and Plant Health Inspection Service, National Veterinary Services Laboratory, Ames, Iowa, USA, for serotyping.

Antibiotic sensitivity

All salmonella strains isolated were tested using a disk diffusion method to determine sensitivity to various antibiotics. Selected colonies from 18–24 h cultures were diluted in sterile 0·85% sodium chloride using the PROMPT™ Inoculation System (Medical Products Division/3M Corp., St Paul, Minn., USA). A sterile swab dipped into the suspension was used to inoculate the surface of Mueller–Hinton agar (Difco) to produce contiguous growth. Sensi-Disc® Microbial Susceptibility Test Discs (BBL) impregnated with 11 different antibiotics were placed on the inoculated surface. The following antibiotics were used: amikacin (30 μg per disk), ampicillin (10 μg), carbenicillin (100 μg), chloramphenicol (30 μg), erythromycin (15 μg), furazolidone (300 μg), gentamicin (10 μg), neomycin (30 μg), polymyxin-B (300 units), tetracycline (30 μg), and triple sulfa (250 μg). Plates were examined after 18–24 h of incubation at 35 °C. Zones of inhibition were measured, and resistance or susceptibility was determined in accordance with the manufacturer's instructions.

Specific procedures

Microbiological surveys were carried out on farms A and B to determine the prevalence and distribution of *Salmonella* spp. in the environment of breeding turtles and hatcheries.

A random sample comprising 197 live turtle hatchlings was obtained from a batch of approximately 40000 condemned from farm B following isolation of *Salmonella* spp. in December, 1988. The affected batch was hatched 6 months previous to the examination and had been maintained under refrigeration (15 °C). Individual live turtles (185) were placed in 400 ml beakers containing 60 ml sterile distilled water. A 2 ml sample of water was removed after 72 h and assayed for *Salmonella* spp. At this time turtles were narcotized with carbon dioxide and killed by decapitation. Using sterile technique, a 0·5 cm^3 portion of liver tissue and a 1 cm length of intestine were pooled in selenite broth for salmonella assay. The yolk sac was collected from each turtle and assayed separately for the presence of *Salmonella* spp.

Records of salmonella assays conducted by an approved certifying laboratory were reviewed. Data were correlated by year and submitter. Salmonella isolates obtained during 1989 were identified to genus level and then referred to the National Veterinary Services Laboratory for serotyping.

Statistical review of sampling protocol

The probability of detecting salmonellosis in a consignment of turtles was calculated for sample sizes ranging from 12 to 200, assuming prevalence rates from

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0·5 to 13·0%. The required sample size was based on the standard normal approximation of the binomial distribution, according to the formula

$$n = \frac{(z^2 * p * q)}{d^2}$$

where n is the sample size, z is the tabulated standard normal value, p is the estimated prevalence rate, q is 1-p, and d is the acceptable difference from the true prevalence rate.

RESULTS

Environmental samples

The results of examination of environmental specimens are indicated in Table 1. *Salmonella* spp. were obtained from pond water (Table 2) on both farms and soil from farm B. No salmonella isolates were obtained from hatchery specimens from either of the two farms.

Live turtle hatchlings

One hundred eighty-five live, refrigerated turtles hatched in December, 1988, were sampled in July, 1989. Twenty-three individual hatchlings were positive for either *S. arizonae* or *S. poona*, representing an overall recovery rate of 12·4% from one or more of the three sites examined (viscera, yolk sac, or water) (Table 3). The isolation rate from pooled liver and intestine (designated 'viscera') was 7·6%, twice that of either yolk sac (3·2%) or water in which turtles were allowed to swim for 72 h (3·2%). A total of 26 salmonella isolates were obtained from the 23 live turtles, since infection of more than one site was present in three individuals. *Salmonella arizonae* was isolated from 1 of 12 dead hatchlings subjected to whole body homogenization.

Salmonella poona was identified more frequently (17/197 turtles) than *S. arizonae* (11/197 turtles) (Table 4). Of the 185 live turtles sampled, one individual excreted both *S. poona* and *S. arizonae* into water and two showed dual infection of viscera. The yolk sac was infected equally with either organism, but *S. poona* was recovered more frequently from water in which turtles were immersed (5/23) than *S. arizonae* (1/23).

Antibiotic sensitivity

All isolates of *S. arizonae* and *S. poona* from turtles derived from farm B were resistant to erythromycin, gentamicin, tetracycline, and triple sulfa. Salmonella isolates from pond water from farm A (*S. arizonae*, *S. cubana*, and *S. newport*) and from farm B (*S. arizonae* and two isolates of *S. poona*) showed similar antibiotic sensitivity patterns. All six isolates were resistant to erythromycin, and the *S. cubana* isolate from farm A was resistant to tetracycline. All isolates were sensitive to amikacin, ampicillin, chloramphenicol, gentamicin, neomycin, nitrofurantoin, polymyxin B, and, with the exception of *S. cubana*, tetracycline.

Records of salmonella isolates supplied to the Louisiana Department of Agriculture and Forestry by the approved certifying laboratory were analysed (Table 5). During 1988, 28 farmers delivered 115 batches for examination. Five

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Table 1. *Environmental samples obtained from farms A and B*

	Farm A	Farm B	Total
Environmental samples			
Water	3/27	3/9	6/36
Soil	0/33	1/20	1/53
Cloacal swab	0/60	0/25	0/85
Feed	0/2	—	0/2
Nest egg surface swab	0/18	0/12	0/30
Nest eggs	0/5	0/2	0/7
Hatchery samples			
Egg washing solution	0/2	—	0/2
Chlorine dip solution	—	0/1	0/1
Antibiotic dip solution	0/4	0/7	0/11
Water in incubation boxes	—	0/16	0/16
Incubator eggs	0/48	0/28	0/76
Eggs before chlorine dip	0/10	0/12	0/22
Eggs after chlorine dip	0/5	—	0/5
Eggs after mechanical washer	0/10	0/10	0/20
Eggs after gentamicin dip	0/10	0/10	0/20
Turtle hatchlings	0/20	0/15	0/35
Hand wash solution	0/1	—	0/1

Table 2. Salmonella *sp. serotypes isolated from samples of pond water*

Farm	Month	Recovery rate	Serotypes
A	July	3/27 (11%)	*S. newport* *S. arizonae* *S. cubana*
B	July/August	3/9 (33%)	*S. arizonae* *S. poona**
	Total	6/36 (17%)	

* Two isolates obtained.

Table 3. *Prevalence of* Salmonella *spp. in hatchling turtles from farm B*

	Number of *Salmonella* spp. isolates	
Sample	Isolates/sampled	%
Viscera	14/185	7·6
Yolk sac	6/185	3·2
Water	6/185	3·2
Individual live turtles*	23/185	12·4
Dead turtles	1/12	8·3
All turtles	24/197	12·2

* Three live turtles showed infection in more than one site.

isolates of *Salmonella* spp. were obtained, representing 4·3% of the total submissions. Four of these isolates were referred to the National Veterinary Services Laboratory for serotyping; three were identified as *S. arizonae* and one as *S. poona*. All four isolates were resistant to erythromycin, gentamicin, tetracycline, and triple sulfa, and sensitive to furazolidone and polymyxin B.

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Table 4. *Relative isolation of* S. arizonae *and* S. poona *from hatchling turtles from farm B*

	S. arizonae		*S. poona*	
Sample	Isolates/infected turtles	%	Isolates/infected turtles	%
Viscera	6/23	26·1	9/23	3·9
Yolk sac	3/23	13·0	3/23	13·0
Water	1/23	4·3	5/23	21·7
Dead turtle homogenate	1/12	8·3	0/12	0

Table 5. *Record of* Salmonella *spp. isolated by approved certifying laboratory*

Year	Number of farmers	Number of submissions	*Salmonella* sp. isolated. number (%)
1986	25	88	0 (0)
1987	24	87	1 (1·2%)
1988	28	115	5 (4·4%)

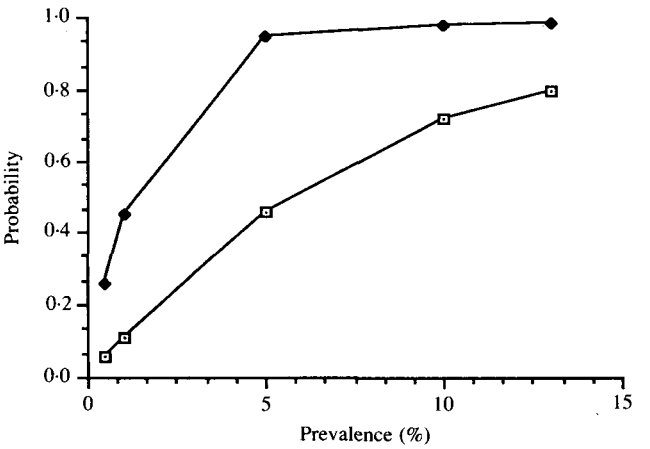

Fig. 1. Probability of detection of colonization with *Salmonella* spp. in a specific batch of hatchlings. ◆——◆, sample size 60; ⊡——⊡, sample size 12.

Statistical evaluation of sampling protocol

The various combinations of prevalence rate, sample size, and probability of detection of salmonella infection in a batch of homogenized turtle hatchlings are depicted in Figure 1. It is apparent that a sample size of 60 turtles will detect at least one positive turtle in a batch with a probability of 0·99, given a prevalence rate of 13%. In contrast, a low prevalence rate of 1% would be associated with a 0·4 probability of detection using the approved sampling protocol.

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DISCUSSION

Surveys on two farms confirmed previous studies which showed that pond water is a principal reservoir of salmonella for turtles. Eggs are contaminated at the time of oviposition by bursal fluid, which is derived directly from pond water.

Eliminating salmonellae from pond water by filtration, chlorination, or other antibacterial measures is not practical given the volume and source of the water. The presence of mud, essential for the survival of turtles during winter hibernation, contributes to the persistence of *Salmonella* spp. in the environment of the pond. The risk of eggs becoming contaminated is enhanced by farming practices, which confine oviposition to a relatively small area of soil adjacent to each pond. Turtles are further constrained in their selection of nest sites by metal screens placed in the laying area to facilitate collection of eggs by farmers.

Subjecting eggs to a rinse in sodium hypochlorite solution to remove adherent soil, followed by immersion in gentamicin solution, has proven effective in reducing, but not eradicating salmonella infection in hatchlings. Improved management practices, including collection of eggs in cleaned baskets, washing eggs on arrival at the hatchery, immersion of eggs in chlorine solution, and elimination of sand and sawdust as incubation media, have all contributed to a reduction in infection. Adoption of hygienic handling of eggs by responsible producers and adherence to regulations intended to eliminate infection have collectively reduced the level of contamination in hatchlings from approximately 40% prior to 1980 to below 1% in 1985.

Immersion in gentamicin solution should theoretically decontaminate eggs and produce hatchlings free of *Salmonella* spp. Variation in pH of treatment solutions and concentration of the antibiotic, improper operation of immersion equipment, presence of organic matter on eggs, and the extent to which eggs absorb water from soil are all variables which may influence the effectiveness of treatment.

Isolates of *S. arizonae* and *S. poona* obtained from live turtles from farm B were resistant to gentamicin, as were the four isolates identified during 1988 by the certifying laboratory. Although gentamicin-resistant *Salmonella* spp. were not identified in water samples from either farm, it is highly probable that contamination of ponds with gentamicin-resistant *Salmonella* spp. will eventually occur. The emergence of drug-resistant serovars calls into question the value of the Louisiana program of immersing eggs in gentamicin solution.

The Louisiana testing protocol requiring submission of 60 turtles is based on the United States Federal regulations framed in 1972. At this time the prevalence of salmonella isolation in batches of hatchling turtles was about 40% [4]. After the introduction of gentamicin treatment, the prevalence rate of *Salmonella* spp. in hatchling turtles was reduced to below 0·5%. The standard formula for calculating sample size shows that 60 turtles would provide only a 0·26 probability of detection of salmonella with a prevalence rate of 0·5%. This fact may be responsible for the observation that batches of turtles certified to be free of *Salmonella* spp. before shipment from Lousiana subsequently were found to be infected with *Salmonella* spp. on arrival in the importing country. As the level of infection increases to 5% the probability of detecting *Salmonella* sp. in a batch of 60 turtles will rise to 0·95. Assuming a 13% prevalence rate, as determined in the

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rejected batch derived from farm B, infection would be detected with a probability of 0·99, given a sample size of 60 turtles.

Stress, including dehydration, will enhance excretion of *Salmonella* spp. by infected turtles [16]. That turtles are subjected to stress after purchase is indicated by a study which showed that the life span of an average pet turtle in 1975 was less than 2 months [3]. High biodensity and stress during transport will lead to intra-batch transmission of infection. Inappropriate management and nutrition subsequent to purchase will increase the probability of dissemination of visceral salmonella infection into the environment.

The epidemiology of salmonella infection at the farm level, the emergence of gentamicin-resistant strains, and the inherent sensitivity of current methods of isolation will all contribute to an increasing frequency of condemnation of batches of turtles submitted for certification.

Despite the introduction in 1983 of equipment to allow immersion of turtle eggs in gentamicin solution under negative pressure, the emergence of resistant strains of *Salmonella* spp. will result in vertical transmission of infection in turtle hatchlings, representing a risk of infection to purchasers. This study, which documents the emergence of gentamicin-resistant strains of *Salmonella* spp. in pet turtles, confirms that immature turtles (*Pseudemys scripta elegans*) are inappropriate pets.

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