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Virulence Determinants *invA* and *spvC* in Salmonellae Isolated from Poultry Products, Wastewater, and Human Sources

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The presence of two virulence foci, *invA* and *spvC*, in *Salmonella* isolates obtained from poultry, wastewater, and human sources was determined. All isolates ($n = 245$) were positive for the *invA* gene sequence. Differences in degree of invasiveness were apparent with the Madin Darby canine kidney cell line, as only 79 of 159 randomly selected isolates (49.7%) tested were invasive at >0.1% of the inoculum, 25% were invasive between 0.1 and 1.0% of the inoculum, and 24.5% were invasive at >1.0% of the inoculum. There was a significant correlation between degree of invasion and source from which the isolate was recovered but no correlation between geographic origin of poultry isolates and degree of invasion. Only 37 of 245 isolates (15.1%) hybridized with the *spvC* DNA probe. All isolates that were recovered from a commercial egg production environment and chicken eggs and whose sequences exhibited homology with the *spvC* gene sequence were determined to be either *Salmonella enteritidis* PT 23 or PT 13. The sequences of few isolates from ceca and none from wastewater or humans demonstrated homology with the *spvC* gene.

Human salmonellosis occurs in a variety of forms, including gastroenteritis, organ focal infection, or systemic febrile infection (20). Salmonellae are widely distributed in nature and are associated with both wild and domesticated animal hosts (14). A number of salmonella serotypes may be transferred to poultry from various sources, such as feedstuffs, breeding flocks, rodents, and wild birds, etc. (2). Non-host-adapted strains, like *Salmonella typhimurium*, *S. enteritidis*, and *S. heidelberg*, are infrequently transmitted transovarially in chickens (33, 36).

The Centers for Disease Control and Prevention first reported a fivefold increase in *S. enteritidis* isolation rates in northeastern and middle Atlantic states between 1976 and 1985 (3). In the United States this epidemic rise in *S. enteritidis* infections has been attributed to grade A shell eggs contaminated via the transovarian route. European countries have since experienced similar egg- and poultry-associated outbreaks (6, 28, 34).

Salmonellae cause disease by invading the intestinal epithelium after ingestion (7, 8, 25). Researchers have studied the internalization of salmonellae, such as *S. typhi*, *S. typhimurium*, and *S. choleraesuis*, in epithelial cells and found that they multiply within formed vacuoles (11, 12, 38). In vitro systems using different cell lines have been used to study the interaction between salmonellae and eukaryotic cells (9, 15, 38). Galan and Curtiss (13) first characterized the *Salmonella* invasion gene *invA*, the first gene in an operon which is thought to trigger the internalization of *S. typhimurium* in cultured epithelial cells. Mutations within this operon render salmonellae incapable of invading Madin Darby canine kidney (MDCK) cells in culture.

Virulence plasmids are one of several *Salmonella* virulence determinants involved in survival and growth in host cells (8). Virulence plasmids are thought to not be involved in the initial interaction between salmonellae and the intestinal mucosa or required for invasion into deeper tissue. However, they enable the organism to persist in the reticuloendothelial cells of liver and spleen (8, 16). They range in size from 50 to 100 kb (18, 22, 26, 27). Gulig et al. (17) identified a 7.8-kb region of a 90-kb *S. typhimurium* virulence plasmid capable of conferring the organism's complete virulence plasmid phenotype. This virulence region is similar to one reported by Williamson et al. (37) for *S. dublin*. This 7.8-kb region encompasses five genes collectively designated *spvRABCD*, for *Salmonella* plasmid virulence. The exact function of *spvC*, formerly known as *virA*, is unknown. However, it seems to increase the growth rate of salmonellae in host cells and affect their interaction with the host immune system (19).

Both invasion and the presence of the virulence plasmid are essential for the full expression of virulence of *S. typhimurium* in mice, which is the model for human typhoid, a systemic disease. The relationship between the presence of both the *invA* and *spvC* genes and the degree of invasiveness among salmonella isolates recovered from diverse poultry-associated sources has not been determined. A comparison of these with strains of human and environmental origins may further clarify the nature of poultry-related human salmonellosis. The purpose of this study was to compare salmonella isolates obtained from commercially processed poultry, eggs and the egg production environment, humans, and wastewater treatment plants for invasiveness in epithelial cells, the presence of the invasion gene *invA*, and the presence of *spvC*.

MATERIALS AND METHODS

Bacterial strains. Salmonellae were isolated from samples obtained from commercially processed spent hens, broilers, and eggs and the egg production environment. Poultry-associated samples included ovaries and oviduct tissue, cecum tissue sections, whole egg contents, and the egg production environment. Poultry processing plants were located in various southeastern, middle Atlantic, and middle western states. Wastewater isolates were recovered from the outfalls of three area municipal wastewater treatment facilities. Human isolates were obtained from the Georgia State Epidemiology Laboratory, Atlanta, Ga. All *Salmonella* isolates were serotyped at the National Veterinary Services Laboratory, Ames, Iowa. *S. typhimurium* SR 11 (χ3181) and *Escherichia coli* HB101 were used as the positive and negative controls, respectively, for the invasion assay, plasmid isolation, and DNA-DNA hybridization (13, 16).

Invasion assay. Tissue culture reagents were obtained from GIBCO Laboratories, Grand Island, N.Y., and Sigma Chemical Co., St. Louis, Mo. MDCK cells were acquired from American Type Culture Collection, Rockville, Md. Cells were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin (100 μg/ml), streptomycin sulfate (100

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TABLE 1. Presence of *spvC* and level of invasiveness of *Salmonella* isolates

Source of isolate	No. of isolates/no. tested (%)		% Invasion		
	spvC positive[a]	Invasive at >0.1% of the inoculum	Mean ± SD	Median	Range
Chicken ovaries	4/28 (14.3)	16/28 (57.1)	1.50 ± 1.67	0.32	0.11–5.30
Chicken oviduct	0/4 (0.0)	2/4 (50.0)	0.89 ± 0.80	0.89	0.17–1.60
Chicken cecum	2/53 (3.8)	12/35 (34.3)	1.50 ± 2.79	0.48	0.10–10.50
Chicken egg	16/16 (100.0)	14/14 (100.0)	2.35 ± 2.03	1.55	0.16–7.00
Egg environment	14/45 (31.1)	24/37 (64.9)	3.20 ± 3.47	1.8	0.10–12.20
Wastewater	0/82 (0.0)	6/24 (25.0)	0.58 ± 0.20	0.58	0.15–0.80
Human	1/17 (5.9)	5/17 (29.4)	0.43 ± 0.19	0.4	0.15–0.74
Total	37/245 (15.1)	79/159 (49.7)			

[a] Number of isolates that were positive by DNA-DNA hybridization for *spvC* in colony blots or by blotting plasmid preparations.

μg/ml), amphotericin (1 μg/ml), and gentamicin (100 μg/ml). Monolayers were grown to confluency (approximately 2×10^5 viable cells per ml) in 24-well culture plates.

The invasion assay of Finlay and Falkow (7) was used with slight modification. Residual antibiotics were removed from cell monolayers by five washes with Eagle's minimal essential medium without antibiotics and incubation overnight. Ten microliters of standing 18-h bacterial culture, in Lennox broth, was added to the monolayer and incubated for 2 h at 37°C in a 5% CO_2 atmosphere. The medium was replaced with Eagle's minimal essential medium containing 100 μg of polymyxin B per liter and 100 μg of gentamicin sulfate per ml and incubated for 1 h at 37°C (29). Monolayers were washed five times with Hank's balanced salt solution to remove antibiotics. Monolayer cells were lysed by adding 0.2 ml of a 1% Triton X-100 solution per well and incubated for 5 min. A total of 0.8 ml of Lennox broth was added, and the cells were incubated at 37°C for 5 min under 5% CO_2. Monolayers were physically disrupted by pipetting, and titers of internalized bacteria were determined on MacConkey agar (Difco, Detroit, Mich.). Inoculum levels were also determined, and percent invasion was calculated by the following formula: (CFU recovered after cell lysis/CFU of inoculum) $\times$ 100. Each invasion assay was run in triplicate along with positive and negative controls.

***invA* and *spvC* probes.** 21-mer primers for *Salmonella spvC* and *invA* gene probes were designed by using published sequences (GenBank accession numbers M64295 and M90846, respectively) and Oligo software (National Biosciences, Plymouth, Minn.). The *spvC* primer sequences were 5′-CGGAAATA CCATCTACAAATA-3′ and 5′-CCCAAACCCATACTTACTCTG-3′, which were predicted to yield a 669-bp product. The *invA* primer sequences were 5′-TTGTTACGGCTATTTTGACCA-3′ and 5′-CTGACTGCTACCTTGCTG ATG-3′, which were predicted to yield a 521-bp product. Primers were prepared with an ABI model 394 DNA synthesizer by the University of Georgia Molecular Instrumentation Laboratory. Genomic DNA was isolated from *S. typhimurium* SR 11 as previously described (35). Probes were prepared by PCR with a Perkin-Elmer model 480 thermocycler with denaturation at 93°C for 1 min, primer annealing at 42°C for 1 min, and primer extension at 72°C for 2 min for a total of 30 cycles. The PCR products were nonradioactively labeled during synthesis with digoxigenin-11-dUTP as per the manufacturer's instructions (Boehringer GmbH, Mannheim, Germany). The PCR products were then purified by affinity chromatography with a Magic PCR Preps DNA purification system (Promega Corp., Madison, Wis.) in order to remove excess primer and digoxigenin-labelled nucleotides. The labelled PCR products were used as probes in DNA-DNA hybridization experiments with colonies or plasmid DNA.

Plasmid DNA isolation. Plasmid DNA was isolated and purified with a Wizard Mini-Prep kit (Promega Corp.). Plasmid DNA was characterized by gel electrophoresis on a 1% agarose gel, according to standard methods (35).

Hybridization. Colonies were grown on brain heart infusion agar and then patched onto nitrocellulose membranes (Micro Separations, Inc., Westboro, Mass.) with sterile toothpicks for the colony blots. The colonies were lysed, and then the DNA was denatured and neutralized (35). For some studies the plasmid preparation was spotted onto nylon membranes (Micro Separations, Inc.) and used for hybridization. The DNA-DNA hybridizations were performed by prehybridization for 1 h at 68°C in 5× SSC [5× SSC is 0.6 M NaCl plus 0.06 M sodium citrate) containing 1% casein, 0.1% *N*-lauroylsarcosine, and 0.02% sodium dodecyl sulfate. Hybridization was conducted at 68°C overnight in buffer containing a boiled (10 min at 100°C) digoxigenin-labelled probe. Enzyme immunoassay for detection of the presence of digoxigenin-labelled DNA was performed by the method of the supplier (Boehringer GmbH).

Statistical analysis. The sources of isolates and presence of the virulence plasmid were correlated by Ryan's procedure (30). A point-biserial correlation was done to correlate the level of invasion and the presence or absence of the virulence plasmid. A one-way analysis of variance was conducted to determine significant differences in the levels of invasiveness of isolates recovered from different states and between invasiveness and sources of poultry isolates.

RESULTS

Probes. The PCR-mediated amplification resulted in the production of products of expected size. The *invA* product was approximately 500 bp and the *spvC* product was approximately 650 bp, as determined by gel migration. Sequence analysis (at least 99% homology with GenBank sequence data) confirmed the identities of the products (data not shown).

Presence of *invA*. The 500-bp *invA* PCR product was used to probe colony blots. All 245 *Salmonella* isolates examined were positive for *invA* by DNA-DNA hybridization. The controls, *S. typhimurium* SR 11 and *E. coli* HB101, were positive and negative, respectively.

Invasion assay. A total of 159 *Salmonella* isolates representing all sources were examined for invasiveness. These data are presented in Table 1. Approximately 50% of all isolates were invasive at <0.1% of the inoculum, 25.5% were invasive between 0.1 and 1.0% of the inoculum, and the remaining 24.5% were invasive at >1.0% of the inoculum.

Multiple serotypes were represented among the isolates. In general, the egg and egg production environment isolates demonstrated the highest percentage of cell invasion of all isolates. Among the chicken oviduct isolates (n = 4), an *S. muenchen* isolate was the most invasive, at 1.6%, and *S. enteritidis* PT 8 was the most invasive (at 5.3% of the inoculum) of the isolates from chicken ovaries (n = 28). A nontypeable *Salmonella* strain was most invasive (10.5%) of those recovered from chicken ceca (n = 53). *S. enteritidis* PT 13 was the most invasive (7.0%) of the isolates recovered from whole egg contents (n = 16). An isolate of *S. enteritidis* obtained from the egg production environment was the most invasive of all the isolates assayed (12.2%).

The wastewater and human isolates (n = 24 and 17, respectively) were the least invasive. An *S. heidelberg* isolate recovered from the wastewater treatment plant outfall showed the highest level of invasiveness, at 0.8%, while of the human isolates, an *S. muenster* isolate was the most invasive (0.74%).

One-way analysis of variance revealed a correlation between invasion level and isolate source. Pairs of sources are listed on the basis of statistical correlation from highest to lowest: eggs and human sources, chicken eggs and wastewater, egg production environment and wastewater, and cecum and egg production environment ($P < 0.05$).

One-way analysis of variance revealed no significant difference in invasiveness for isolates from any of 10 states ($P <$ 0.05), indicating that no one geographic area has a greater preponderance of invasive *Salmonella* serotypes in poultry.

Presence of the *spvC* gene. As determined by using the 650-bp PCR product as a probe, 37 of 245 isolates tested by

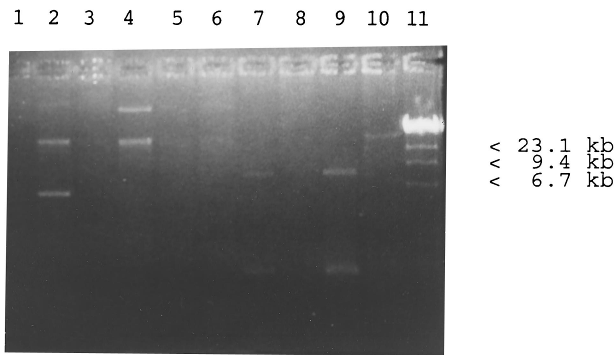

FIG. 1. Agarose gel electrophoresis of 11 *Salmonella* isolates. Lanes (organism sources are in parentheses): 1, *S. heidelberg* (ovaries); 2, *S. typhimurium* (ovaries); 3, *S. enteritidis* PT 13 (chicken egg); 4, *S. enteritidis* (egg processing environment); 5, *S. enteritidis* PT 23 (ceca); 6, *S. enteritidis* PT 23 (ceca); 7, *S. enteritidis* PT 28 (ceca); 8, *S. albany* (ceca); 9, *S. enteritidis* PT 28 (ceca); 10, *S. enteritidis* PT 13a (ceca); 11, standard (*Hin*dIII digestion of lambda). Note that lanes 2, 4, 5, and 6 show a large-molecular-size band which migrates more slowly than the 23.1-kb linear standard.

colony blotting (15%) contained *spvC*-homologous regions (Table 1). The majority of those positive isolates (81%) originated from egg contents or the egg production environment. All salmonellae recovered from eggs were *S. enteritidis* and were positive for the presence of the *spvC* gene sequence. Fourteen of 45 isolates (31%) recovered from the egg production environment, which included swabs of egg collection equipment and manure trays, hybridized with the *spvC* probe. *S. enteritidis* isolates were either PT 23 or PT 13. In contrast, no wastewater isolates and few cecal and human isolates (3.8 and 5.9%, respectively) demonstrated homology for the *spvC* gene.

Six of 19 isolates tested demonstrated the presence of a large-molecular-size plasmid (Fig. 1, lanes 2, 4, 5, and 6). Eight of these gave positive signals with the *spvC* DNA probe. Analysis of plasmid DNA revealed three *spvC*-positive isolates that were negative by the colony blot method. This could suggest a low copy number of plasmid DNA in the colony blot. No large-molecular-size plasmids were visualized for strains which were *spvC* negative. Negative results obtained by gel electrophoresis may indicate that the plasmid DNA had integrated into the chromosome and was not present in the plasmid preparation. Jones et al. (26) reported that the *Salmonella* virulence plasmid could integrate into the chromosome, resulting in a failure to express the virulence phenotype. Therefore, colony blotting would have detected *spvC* had this occurred.

The presence of the virulence plasmid was associated with the strain source at a P value of <0.001 by using Ryan's statistical procedure for pairwise comparisons between portions (30). The degree of invasion was significantly correlated with the presence of the virulence plasmid at a P value of <0.001. By using the point-biserial correlation, the coefficient of correlation was found to be 0.54.

DISCUSSION

While the transmission of salmonellae via poultry products, particularly egg-associated *S. enteritidis* to humans, is well documented (4), little information on the characterization of salmonella isolates from commercial poultry processing and egg production exists. Thus far only *S. enteritidis* has been implicated in food-borne illness associated with commercially produced eggs. However, our laboratory has recovered multiple *Salmonella* serotypes from the broader poultry and egg processing environment (1, 5) which have been characterized in this study. Penetration of host epithelial barriers is of particular importance in the pathogenesis of salmonellosis when it occurs as the cause of food-borne illness. Cell culture models represent an experimental system in which in vivo epithelial cell barriers can be produced under defined conditions to evaluate bacterial invasion in vitro (10, 32).

All isolates ($n = 245$) representing multiple serotypes from all sources were positive for the *invA* gene sequence. There were, however, differences in invasion levels. All isolates from chicken eggs were invasive at $>0.1\%$ of the inoculum. Sixty-five percent of those from the egg production environment and 57% of those from ovaries were invasive at this level, while invasiveness among isolates from other sources ranged from 25 to 35%.

A role for the virulence plasmid in salmonellosis has been reported by several researchers. Gulig and Curtiss (18) demonstrated an association between the presence of the virulence plasmid in *S. typhimurium* and virulence in mice. Similarly, Hovi et al. (24) claimed the same for a 36-megadalton plasmid in *S. enteritidis* in mice. Others have concluded that the gene encoding virulence factors for human enterocolitis were on the bacterial chromosome and not on the serovar-specific plasmid (21, 23).

In this study, only 37 of the 245 isolates (15.1%) had sequences that hybridized with the *spvC* probe. Montenegro et al. (31) reported that nearly 100% of the strains isolated from animal organs and human blood were positive for the virulence plasmid. In our study, the isolates represent diverse sources and a variety of serotypes, which may account for the lower percentage of *spvC*-positive strains found. All of the egg isolates carried the virulence plasmid and were more invasive, while few recovered from chicken ceca were *spvC* positive. No wastewater isolates, which represent diverse sources, contained the plasmid. Only one human isolate carried the virulence plasmid. It seems that *spvC* does not play a critical role in establishing the typical gastroenteritis caused by salmonellae in humans.

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