ORIGINAL ARTICLE

Evaluating Wildlife as a Potential Source of *Salmonella* serotype Newport (JJPX01.0061) Contamination for Tomatoes on the Eastern Shore of Virginia

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Impacts

- This study produced a variety of different *Salmonella* serotypes and patterns from wildlife specimens, some of which have been historically associated with outbreaks from tomatoes grown in Virginia.
- Birds were responsible for almost half of the *Salmonella*-positive samples in this study.
- Certain *Salmonella* serotypes may be highly resistant to extreme weather conditions such as heat and desiccation.

Keywords:
Salmonella Newport; wildlife; tomato; virginia; ELISA; PFGE

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Summary

Eastern Shore of Virginia red, round tomatoes contaminated with *Salmonella* serotype Newport pattern JJPX01.0061 have been a source of several multistate outbreaks within the last 10 years. No source of the contamination has yet been identified. The goal of this study was to evaluate wildlife as a potential source of contamination. Faecal samples from deer, turtles and birds were collected between November 2010 and July 2011 from seventeen locations on the Eastern Shore of Virginia. A total of 262 samples were tested for the presence of *Salmonella* using an enzyme-linked immunosorbent assay (ELISA). A total of 23 (8.8%) samples tested positive for *Salmonella* spp. and were further characterized by serotyping and pulsed-field gel electrophoresis (PFGE) subtyping. Overall, twelve serotypes were identified, including *Salmonella* serotype Javiana, another common serotype associated with tomato-related outbreaks. Only one avian sample collected in July 2011 was determined to be positive for *S.* Newport pattern 61. This sample was collected from the ground at a site where birds, mostly gulls, were congregating. Although many of the avian samples from this site were dry, the site yielded eleven positive *Salmonella* samples. This suggests that certain *Salmonella* serotypes may persist in the environment despite extreme conditions. The recovery of one Newport pattern 61 isolate alone does not yield much information regarding the environmental reservoirs of this pathogen, but when combined with other data including the recovery of several isolates of Javiana from birds, it suggests that birds might be a potential source of *Salmonella* contamination for tomatoes on the Eastern Shore.

Introduction

Foodborne *Salmonella* outbreaks have been traditionally associated with poultry, but produce outbreaks are becoming increasingly common. Recently, it was estimated that 13% of outbreaks in the United States may be attributed to produce contaminated with foodborne pathogens (Hanning et al., 2009). A wide variety of produce including tomatoes, sprouts, cantaloupe and lettuce have been implicated in recently reported outbreaks. Produce can be contaminated through a number of routes in the field, including, but not limited to, surface or irrigation water, faeces from livestock or wildlife, raw or poorly composted manure, farm equipment and human workers. Tomatoes seem particularly susceptible to *Salmonella* contamination. Previous studies have shown that *Salmonella* can either colonize or proliferate on the surface of a tomato depending on the serotype or the environmental conditions (Hanning et al., 2009; Pao et al., 2012).

Since 1998, over 15 *Salmonella* outbreaks causing more than 2000 confirmed illnesses have been linked to tomatoes. The number of cases could be closer to 60 000 since the CDC estimates that there are potentially 29.3 people ill for every confirmed case of *Salmonella* (CDC, 2011). Many of these outbreaks have been associated with fresh or fresh-cut tomatoes from Florida and Virginia. In Virginia, the Eastern Shore has been associated with five outbreaks and almost 850 confirmed illnesses.

The Eastern Shore of Virginia is a narrow strip of land made up of two counties: Northampton and Accomack (Fig. 1). It is attached to the south-eastern tip of Maryland and separated from the rest of Virginia by the Chesapeake Bay and is bordered on the eastern edge by the Atlantic Ocean. The Eastern Shore of Virginia is comprised of mostly rural, agricultural communities that produce chickens and grow crops such as potatoes, peanuts, soybeans and tomatoes throughout the Shore. Tomato production on the Eastern Shore contributes to making Virginia one of the top producers of tomatoes in the nation.

Outbreaks occurring on the Eastern Shore have been associated with one particular serotype, *Salmonella* serotype Newport. *S.* Newport is the third leading cause of *Salmonella* outbreaks in the United States (CDC, 2012). Pulsed-field gel electrophoresis (PFGE) or DNA 'fingerprinting' has further subtyped the strain as *S.* Newport pattern JJPX01.0061 (denoted as *S.* Newport pattern 61). The

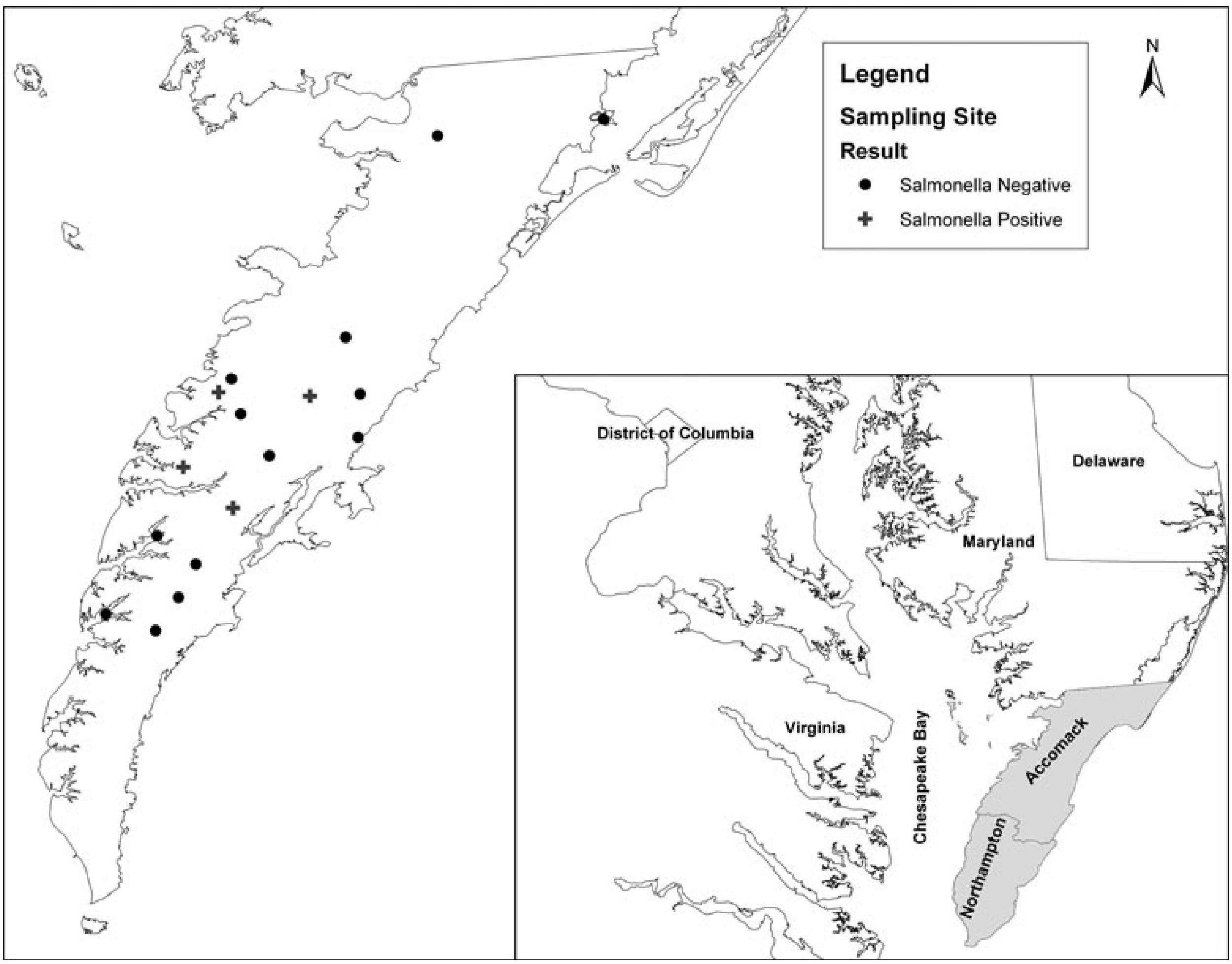

Fig. 1. Map showing the location of Accomack and Northampton counties in Virginia. The towns where the 17 sampling sites for *Salmonella* serotype Newport pattern JJPX01.0061 took place are shown on the larger map of the two counties.

identification of this PFGE pattern in all five outbreaks related to Eastern Shore red, round tomatoes is suggestive of a common source for *S.* Newport contamination on the Eastern Shore. The source of the *Salmonella* involved in the outbreaks has not been found, but irrigation pond water sampled in 2005 did yield *S.* Newport pattern 61 (Greene et al., 2008).

It has been speculated that the source of the *S.* Newport pattern 61 might be wildlife. During a joint Food and Drug Administration, Virginia Department of Health and Virginia Department of Agriculture and Consumer Services investigation into the source of the outbreaks, wildlife or evidence of wildlife (faeces and/or tracks) was observed in tomato fields. Documents from the investigation noted that turtles and waterfowl, primarily Canada geese, were seen in ponds that were used for irrigating the tomato plants (Greene et al., 2008). Many of the species observed in the report (geese, gulls and deer) have the ability to spread *S.* Newport in the environment.

Materials and Methods

Deer, geese, ducks, gulls and turtles were chosen to be part of the study to determine the potential role of wildlife in harbouring or spreading *S.* Newport pattern 61 on the Eastern Shore. Prior to sampling, sample size was calculated for all wildlife groups, except turtles, using the Statcalc function in the software program EPI INFO (version 3.4.2; Centers for Disease Control and Prevention, Atlanta, GA, USA). A sample size of 73 was calculated for each group using the following parameters: expected frequency of 5%, worst acceptable level of 0% and confidence level of 95%. The expected frequency of 5% was chosen based on the literature review regarding the prevalence of *Salmonella* in various wildlife species (Fallacara et al., 2001; Millán et al., 2004; Branham et al., 2005; Renter et al., 2006; Jijón et al., 2007). It was estimated that only 20 turtles would be caught during the study period because few turtles were observed on the properties where tomatoes were grown. A scientific collection permit (#040746) was obtained for this study from the Virginia Department of Game and Inland Fisheries prior to sample collection.

Sampling commenced at seventeen sites in Accomack and Northampton counties on the Eastern Shore of Virginia between November 2010 and July 2011 (Fig. 1). Locations and sampling dates were dependent on the animals being sampled. Deer samples were collected from hunter check stations during the 2010 fall hunting season. Waterfowl samples were collected from birds that were taken at various waterbodies on the shore between December 2010 and January 2011. Turtles were sampled from ponds on tomato farms in May and July of 2011. Gulls and other birds were sampled from one particular Accomack County site in May and July of 2011; this site is characterized by a tomato field where multiple bird species were seen and a property across the street where gulls congregated and is bordered by mixed agricultural-use fields as well as wooded properties.

Faecal material from deer, turtles and waterfowl was collected by swabbing (Catch-All™; Sample Collection Swabs, Epicentre, Madison, WI, USA) the rectum or cloaca of either hunter-killed animals (deer, waterfowl) or live caught animals using hoop nets (turtles). In addition to swabbing turtle cloacae, the biofilms on turtle carapaces were also swabbed to improve chances of pathogen recovery (Richards et al., 2004; Gaertner et al., 2008). Samples from other birds were taken by swabbing faecal material left on concrete surfaces, fields or plastic covers on tomato fields. All samples were placed in Cary-Blair transport media and transported by state vehicle to the laboratory at Virginia State University. As Cary-Blair is stable at room temperature, no special packaging was used.

Each faecal or carapace sample (≤1 g) was pre-enriched in 99 ml of buffered peptone water at 36°C for 20 h, followed by enrichment in Rappaport–Vassiliadis (RV) broth at 42°C for 18 h and post-enrichment in mannose (M) broth at 36°C for 7 h before the *Salmonella* enzyme-linked immunosorbent assay (ELISA; Tecra, Frenchs Forest, Australia) was performed (Pao et al., 2005; Tecra International Pty. Ltd, 2005). As enrichment steps were included prior to testing with the ELISA, the assay could detect as little as one *Salmonella* cell per sample (Pao et al., 2005). For ELISA-positive samples, RV and/or M broths were streaked on xylose–lysine–deoxycholate agar (Difco, Sparks, MD, USA.) for isolation. Typical colonies (red colonies with or without black centres) were isolated, and at least one isolate was identified to genus level using Gram staining and API 20 E® test strip (bioMérieux, Marcy I'Etoile, France).

Pulsed-field gel electrophoresis(PFGE) was performed according to standardized procedures developed by the CDC (CDC: Foodborne and Diarrheal Diseases Branch, 2003) Briefly, cell suspensions were prepared and adjusted to a turbidity equivalent of a 3.0 McFarland using cell suspension buffer consisting of 100 mM Tris, pH 8 and 100 mM EDTA, pH8. Cell suspensions were mixed 1 : 1 with 1.2% molecular-grade agarose containing 0.1 mg/ml proteinase K and cast into plug moulds. Bacteria-containing agarose plugs were subjected to cell lysis at 56°C for 1.5 h in 50 mM Tris, pH 8; 50 mM EDTA, pH 8, containing 1% sarcosine and 0.1 mg/ml proteinase K. Plugs were washed 2X in MilliQ water and 4X in TE buffer (10 mM Tris, pH 8; 1 mM EDTA, pH 8) at 50°C with 70 rpm agitation. Agarose-embedded DNA was digested with 50 U of the restriction endonuclease *Xba*I (New England Biolabs, Ipswich, MA, USA) for approximately 3 h in a water bath at 37°C. The restriction fragments were separated by electrophoresis in 0.5X Tris–borate–EDTA buffer at 14°C for 19–19.5 h using a Chef Mapper XRS electrophoresis system (Bio-Rad, Hercules, CA, USA) with pulse times of 2.16–63.8 s. The gels were stained with ethidium bromide, and DNA bands were visualized with a ChemiDoc XRS (Bio-Rad). *Salmonella* serotype Braenderup H9812 was used as the control strain (Hunter et al., 2005). Interpretation of DNA fingerprint patterns was performed using BIONUMERICS 5.1 software (Applied Maths, Austin, TX, USA). The banding patterns were compared using Dice coefficients with a 1.0–1.5% band position tolerance. After testing was completed by PFGE, isolates were serotyped according to the Kauffman and White scheme, using somatic (O) and flagellar (H) antigens (Brenner and McWhorter-Murlin, 1998; Brenner et al., 2000).

Results

A total of 262 samples were collected during the sampling period. Of the targeted animal groups sampled during the study, only deer (n = 73) and turtles (n = 31) met or exceeded the quota set forth at the beginning of the study. The 31 turtles that were sampled during the study yielded 57 samples because both the cloacae and the carapace of the turtles were swabbed. Ducks (n = 72), geese (n = 7) and gulls (n = 29) did not meet the sample size assigned to these groups.

Twenty-four ‘other’ faecal samples were collected as part of the study. Over half of these ‘other’ samples were classified as avian in origin because the samples were collected from a field where mainly gulls and a few other bird species intermingled, making the designation of the sample species origin uncertain. Opportunistic samples were also included in the ‘other’ group. Faecal samples from one snake and two horses were included in the study because they can also be carriers of *Salmonella*. Three faecal samples of mammalian origin from a tomato field were also tested for *Salmonella*.

Of the 262 samples tested for *Salmonella*, 23 samples (8.8%, 95% CI: 5.3–12.3%) were *Salmonella* positive. Twelve serotypes, including *S.* Newport, and eight different PFGE patterns were identified (Table 1). One *S.* Newport pattern 61 sample from a bird was identified during the study. Javiana, another common serotype associated with tomatoes, was the serotype isolated most frequently identified (6/23; 26.1%) during the study. Several of the identified isolates produced PFGE patterns that did not match other PFGE patterns previously identified in Virginia in the past 3 years.

Overall, 15 of the positive *Salmonella* samples (11.9%; 95% CI: 6.2–17.6%) came from birds, six positive samples (10.5%; 95% CI: 2–18.5%) came from turtles, one positive sample (100%; 95% CI: 33.3–100%) came from the snake and one positive sample (33.3%; 95% CI: 0–86.4%) was of mammalian origin. Gulls and those samples that could only be identified as avian in origin, henceforth known as the non-waterfowl group, yielded more *Salmonella* isolates and had a significantly higher *Salmonella* prevalence (14/47; 29.8%; 95% CI: 16.7–42.9%, $P < 0.01$) as compared to hunter-killed waterfowl (1/79; 1.3%; 95% CI: 0–3.8%). Neither geese nor deer produced positive *Salmonella* results.

Eleven sites in Accomack County (188; 71.8%) and six sites in Northampton County (74; 28.2%) were sampled during the study. Of the seventeen sites sampled on the Eastern Shore for *Salmonella*, only four sites yielded positive results. Three of these sites were in Accomack County, while only one site was in Northampton County. The majority of positives (15/23; 65.2%) and hence serotypes were produced by the mixed-use Accomack County site from which gulls and other birds were sampled. At the other three sites, only one or two serotypes were identified.

Table 1. *Salmonella* serotypes and pulsed-field gel electrophoresis (PFGE) patterns for wildlife specimens collected on the Eastern Shore of Virginia

Salmonella serotype	PFGE pattern	Number of samples	Source
Banana	Unique Pattern[a]	2	Avian
Blockley	Unique Pattern	1	Gull
Group T	Unique Pattern	1	Snake
I 4,5,12:i:-	JPXX01.0621	3	Turtle
Javiana	JGGX01.0141	1	Mammal
Javiana	JGGX01.0012	3	Gull
Javiana	Unique Pattern	2	Gull
Mbandaka	Unique Pattern	2	Gull
Newport	JJPX01.0061	1	Avian
Norwich	TDTX01.0035	1	Avian
Senflenberg	Unique Pattern	1	Avian
Thompson	JP6X01.0236	3	Turtle
Typhimurium	JPXX01.0302	1	Duck
Typhimurium var 0 5-(Copenhagen)	JPXX01.1283	1	Gull

[a]Unique Pattern means PFGE pattern did not match any PFGE patterns in the CDC PulseNet National Database based on a 500-day window.

Discussion

Overall, 23 samples (8.8%) were positive for *Salmonella*, which is a slightly higher rate than the predicted prevalence (5%) used for calculating sample size. The higher prevalence is probably due in part to the large number of *Salmonella* isolates coming from non-waterfowl species. When the prevalence for the non-waterfowl group is compared with previously published numbers for gulls in the United

States, the prevalence is also higher than that documented in other studies (Kinzelman et al., 2008; Stoddard et al., 2008). All of the positive non-waterfowl *Salmonella* isolates came from one site in Accomack County.

Javiana was the most frequently isolated serotype, with five samples obtained from the non-waterfowl group and one sample from an unknown source. However, all six of these isolates came from the same location on the Eastern Shore. *S.* Javiana has been implicated in several tomato foodborne outbreaks since the early 1990s, and it has also been isolated from previous tomato farm inspections (Greene et al., 2008). Public information relating to *S.* Javiana PFGE patterns for the *S.* Javiana-related tomato outbreaks or for farm inspections was not available; therefore, PFGE pattern comparisons could not be performed as part of this study.

In addition to having most of the *Salmonella*-positive isolates of all wildlife groups tested, the non-waterfowl group produced two PFGE patterns of interest: *S.* Newport pattern 61 and *S.* Javiana JGGX01.0141 (denoted *S.* Javiana pattern 141). *S.* Javiana pattern 141 is of special interest to the Virginia Department of Health because several suspected outbreaks involving this pattern have occurred in Eastern Shore residents over the last few years. While finding these outbreak patterns in animals is an important first step in determining their origin, causality cannot be attributed; the small number of isolates recovered in this study limits generalizability of the data, and it is difficult to ascertain whether the birds are picking up *Salmonella* in the environment or the birds are the source of the environmental contamination. However, the isolation of these patterns indicates that more targeted research and sampling need to be done on birds, particularly non-waterfowl birds including gulls, to determine what role these animals play in foodborne outbreaks caused by the consumption of *Salmonella* contaminated tomatoes.

One interesting finding of this study was that several of the samples collected from the site in Accomack County that produced all the non-waterfowl positives were severely dried when collected. These positive samples, including the *S.* Newport 61 sample, were collected from a plastic sheet covering a tomato field under a hot sun in July. This suggests that certain *Salmonella* serotypes might be resistant to extreme weather conditions such as heat and desiccation. *Salmonella* are able to survive under difficult circumstances (Uesugi et al., 2007; Gorski et al., 2011), so it is disheartening to know that *Salmonella* contamination of tomatoes may persist in the environment indefinitely despite good agricultural practices. Effective control measures (e.g. fruit washing and cold storage) for post-harvest, processing and food service operations should be utilized in conjunction with good agricultural practices to prevent future outbreaks.

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