



**CPS 2012 RFP
FINAL PROJECT REPORT**

Project Title

Science-based evaluation of risks associated with wildlife exposure for contamination of irrigation water by *Salmonella*

Project Period

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Objectives

- 1.) *Determine genetic relatedness of Salmonella strains isolated from wildlife relative to isolates from irrigation water and sediments in the same geographic region vs. strains from other sources in our database.*
- 2.) *Validate genetic diversity and evaluate virulence potential by pulsed field gel electrophoresis using the PulseNet protocol (Jay-Russell) and by allelic variation of selected genes.*

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Abstract

Salmonella enterica is distributed throughout the environment and can be recovered from various wildlife reservoirs; however, the risks to human health imposed by exposure to strains from these sources are not clear. Different animal host species appear to be associated with different strains of *Salmonella* and are likely to represent a range of disease potential in humans. We proposed to examine *Salmonella* strains recovered from wildlife, irrigation pond water, and sediments sources in order to make genetic comparison to strains derived from human disease. Environmental isolates were collected from the same geographic location, which encompassed 10 ponds in southwest Georgia. Evaluation of disease potential of these strains was accomplished by examining these strain and additional strain collections using PCR-based rapid diagnostics, pulsed field gel electrophoresis (PFGE) analysis, DiversiLab repetitive PCR based typing (rep-PCR), and whole genome sequencing (WGS). Strains were also examined for antibiotic resistance. This research is now linked through WGS to the FDA-sponsored Next Generation Sequencing Project. Thus, the strains collected from this research are now part of a much larger database, which would also include USDA, FDA, and CDC salmonella collections of clinical isolates. This project is integrating these data nationwide and internationally in order to eventually provide a large public genomic database that can be used for source-tracking outbreaks strains. University of Florida is the southeastern representative of this project, and strains from several CPS-funded projects recovered in both Florida and Georgia are now a part of this collection. One of the goals for this research is to establish genetic markers that define the regional distribution and evolution of *Salmonella* from produce. Outcomes could also provide more rapid and accurate assessment of food products implicated in outbreaks in order to prevent the needless broad-based recalls that impact an entire industry. Additionally, these data shed light upon on potential risk imposed by exposure of wildlife to irrigation ponds and provide science-based data for establishing policy and management strategies for irrigation ponds.

The work plan for Objective 1 proposed to marry data for *Salmonella* genotyping based on rep-PCR of isolates from water and sediment samples of irrigation ponds (Wright Lab, 2010-2013) with a survey of *Salmonella* isolates derived from wildlife collected at the same ponds and genotyped by PFGE (Jay-Russell Lab in collaboration with George Vellidis and Lora Smith). These samples provide a strain collection that included > 2000 strains along with the numerous environmental parameters that were recorded from each sample site. PFGE analysis has been completed for a total of 380 strains of *Salmonella* either as part of this study or from prior research. DiversiLab rep-PCR has been completed on a total of 380 strains, and we have sequenced 248 *Salmonella* genomes.

For objective 2 comparative rep-PCR analysis for strains from environmental vs. clinical origin was used to evaluate virulence potential of pond- and wildlife-associated strains. Overall strains from water and sediments (50%) were much more likely to cluster with clinical isolates than were strains from wildlife sources (10%). PFGE and WGS were used to validate the DiversiLab rep-PCR analysis of genetic diversity and relatedness of strains from irrigation ponds in comparison to strains from clinical sources.

Background

Salmonella is the most common bacterial foodborne pathogen in the United States, causing an estimated 1.2 million cases per year (Scallan et al. 2011) or 16.42 illnesses per 100,000 persons (Gilliss et al. 2013). Salmonellosis is also associated with the largest annual incidence of hospitalizations (2,284) and deaths (33) (Gilliss et al. 2013). Furthermore, no significant change in incidence of *Salmonella* infection has occurred since the start of surveillance during 1996-1998 (Gould et al. 2013, Gilliss et al. 2013). The genus *Salmonella* includes two species, namely *S. enterica* and *S. bongori*, with *S. enterica* contributing 99% of reported human *Salmonella* isolates. Over 2500 serotypes had been identified, and around 60% belong to *S. enterica* (CDC 2011). Serotypes Typhi and ParatyphiA cause more serious disease, namely typhoid fever, while other serotypes cause nontyphoidal gastroenteritis. *Salmonella* serotypes Enteritidis, Typhimurium, and Newport account for about half of culture-confirmed *Salmonella* isolates reported by public health laboratories to the National *Salmonella* Surveillance System (CDC 2011). The proportion of *Salmonella* infections that were Enteritidis increased markedly from 1980 to 1995, but declined 30% from 1996 to 2006. Typhimurium has been the most common serotype since 1997, but the proportion of *Salmonella* infections that were Typhimurium decreased 28% from 1996 to 2006. Resistance to at least five antimicrobial agents is common in *Salmonella* Typhimurium. *Salmonella* Newport also increased markedly since 1995, and it is now the third most frequent serotype. *Salmonella* Newport strains resistant to at least 7 antimicrobial agents have been identified in isolates from humans (CDC, 2011).

Salmonellosis is increasingly associated with produce contamination (Gould et al. 2011, Hanning et al. 2009, Danyluk et al. 2007). The number of disease cases per outbreak for produce is now greater than that for other food products (Franz and van Bruggen 2008). Irrigation water has been attributed as a potential source of pre-harvest contamination (Winfield and Groisman 2003, Barak and Liang 2008), with one of the most significant *Salmonella* outbreaks related to fresh produce traced to Jalapeño and Serrano peppers that were contaminated by irrigation water (Behravesh et al. 2011, Mody et al. 2011). Irrigation water may play an important role in contaminating soil and vegetables (Ijabadeniyi et al. 2011, Barak and Liang 2008, Winfield and Groisman 2003), as *Salmonella* is ubiquitously distributed, and the static nature of some irrigation sources may sustain persistent populations (Gaertner et al. 2009, Haley et al. 2009, Cooley et al. 2007, Rajabi et al. 2011). Rivers and ponds provide natural habitat for a variety of wildlife, such as reptiles, amphibians, and birds, which are all known to harbor *Salmonella* and therefore may also serve as reservoirs for this pathogen (Gorski et al. 2013, Reche et al. 2003, Pflieger et al. 2003). Presumably, aquatic systems become contaminated with *Salmonella* through the introduction of fecal material of infected animals (Plusquellec et al. 1994, Ijabadeniyi et al. 2011, Pachepsky et al. 2011). Once present, the pathogen may become established in these environments. *Salmonella* from irrigation sources can adhere to plants and survive for long periods of time (Barak et al. 2009). For example, experimentally introduced *Salmonella* was detected up to 63 days and 231 days on lettuce and parsley, and up to 84 and 203 days on radishes and carrots (Islam et al. 2004). *Salmonella* in drip irrigation water produced detectable contamination of parsley for up to 21 days after the last irrigation event (Lapidot and Yaron 2009). Sprinkler irrigation of contaminated water directly deposited pathogens on aerial tissue, whereas transfer of pathogens from drip irrigation is presumed to occur largely through contact of aerial tissue with the ground or through splashes with rain and irrigated (Girardin et al. 2005).

Uptake through the roots and at the root-shoot interface has also been documented (Klerks et al. 2007).

Periodic testing for generic *Escherichia coli* is now required for agricultural water intended for pre-harvest contact with the edible portion of fresh produce as described in the Produce Safety Rules contained within the 2013 Food Safety Modernization Act (FSMA) require (FDA 2013). Examples of agricultural water include irrigation water that is directly applied to the harvestable portion of a crop, water used for preparing crop sprays, and water used for washing or cooling harvested produce. Farmers are currently responsible for keeping ponds free of debris, trash and domesticated animals. Testing for water associated with pre-harvest include the following: **No more than 235 colony forming units (CFUs) generic *E. coli* per 100 ml for a single water sample, and a rolling geometric mean of five samples of no more than 126 CFU per 100 ml.** Unfortunately, fecal indicator bacteria may not provide reliable estimates of *Salmonella* contamination due to the greater resistance of this pathogen to the stressful conditions associated with environmental reservoirs relative to indicator organisms (Pianetti et al. 2004, Polo et al. 1998). Risks associated with the agricultural exposure to *Salmonella*-contaminated irrigation water have not been systematically evaluated, primarily because pathogen detection methods have lacked the sensitivity needed to detect low levels of *Salmonella* that are generally present in aquatic environments (Escartin et al. 2002, Madsen 1994). Standard methods for recovery and enumeration of *Salmonella* generally include a most probable number (MPN) enrichment in order to increase sensitivity and sample size. Selective agars are used to screen for isolation of presumptive positive colonies, which are then confirmed by biochemical or molecular assays, such as the polymerase chain reaction (PCR) (Haley et al. 2009, Jenkins et al. 2008, Rajabi et al. 2011, McEgan et al. 2013). These methods are labor intensive and may require 3-5 days to complete. Thus, time-efficient and cost-effective detection methods for *Salmonella* from irrigation pond samples are needed for risk assessment and potential monitoring.

Although irrigation water is a recognized risk factor for contamination of produce, the impact of wildlife as a source for foodborne pathogens remains uncertain. Some growers have resorted to using ground water as the sole source for irrigation reservoirs or have cleared vegetation in an attempt to exclude wildlife from these reservoirs. However, these strategies may reduce biodiversity and thus increase the potential for survival of pathogens in irrigation ponds. In order to address controllable risks for contamination of produce by potential vectors for pathogen it is necessary to understand the distribution of these pathogens in wildlife, their virulence potential and relative capacity for transfer to irrigation sources. This current research leveraged results from two previously funded CPS projects, namely “Science-based evaluation of regional risks for *Salmonella* contamination of irrigation water at mixed produce farms in the Suwannee River watershed” (Wright, PI) and “Evaluation of amphibians and reptiles as potential reservoirs of foodborne pathogens and risk reduction to protect fresh produce and the environment” (Jay-Russell, PI). These studies investigated some of the same irrigation ponds on multiple small farms with various vegetable and fruit crops in the upper Suwannee River Watershed in Georgia. This region was selected because it is a "hot spot" for environmental sources of salmonellosis with case rates that were 1.5 times higher than the national average (CDC, 2009). *Salmonella* isolates were recovered from water and/or sediment samples (n>2000) at all ponds (n=10) examined and from 15 (37.5%) of 40 toad, 23 (18.4%) of 125 turtle, 2 (40%) of 5 bivalve samples taken from a subset (n=5) of these ponds (Wright and Jay-Russell, CPS Final Reports).

Salmonella densities directly correlated with water temperature, although we found some correlation of *Salmonella* distribution with fecal coliform and *E. coli* indicators, results were not consistent and *Salmonella* was frequently detected when indicator levels below the recommended standards described above.

The goal for the present research was to establish a phylogenetic database for *Salmonella* accumulated from various studies on irrigation ponds and associated wildlife, including the previously described *Salmonella* isolates from the Suwannee River and irrigation ponds in Georgia and Florida (Rajabi et al., 2011), as well as outbreak-associated strains provided by the Florida Department of Health and the Florida Department of Agriculture and Consumer Services. These genetic analyses have established a regional DiversiLab rep-PCR library, and these data were validated by other approaches, namely, pulsed field gel electrophoresis and whole genome sequencing. Thus, the research has now established regional genetic profiles for *Salmonella* strains based on source and geographic location. The potential risks posed by *Salmonella* in agricultural systems was addressed by considering the genetic relationship of isolates from agricultural sources to pathogenic organisms that are derived from CDC and FDA investigations of *Salmonella* outbreaks throughout the U.S. Serology and molecular typing methods do not adequately discriminate the virulence potential of environmental isolates or their relationship to outbreak lineages. Recent advances in next generation sequencing technologies offer the promise of whole genome sequence analysis as a feasible approach to outbreak investigation and virulence potential prediction (Leekitcharoenphon et al., 2014). Therefore, these studies were linked to FDA efforts to establish a nationwide strain base for evaluation of the geographical distribution of *Salmonella* strains. The ultimate goal will be to develop predictive models that can be used to implement improved agricultural practices and education programs that will reduce produce contamination.

Research Methods and Results

Methods:

Sample collection from irrigation ponds. Water and sediment samples were collected from 10 irrigation ponds on farms within a broad region of the upper Suwannee River Watershed in southern Georgia, as describe previously (Gu et al., 2013; Gu et al., 2013; Wright et al., CPS Final Report, 2010-2012). All 10 ponds were sampled monthly for 12-month period from March 2011 to February 2012; beginning March 2012-February 2013, more intensive sampling efforts focused on the 5 ponds (LV, MD1, NP, CC2 and SC). Characteristics of each pond including size, depth, drainage areas, irrigation types, water intake, associated vegetation, crops and wildlife were recorded along with numerous physical/chemical/biological parameters. Two water samples (10 L) were collected from each pond at locations that were close to the pumps near the irrigation water intake level and at two depths, either at the surface (WAT) or at 50 cm below the surface (WAT_B). Two wet sediment samples (1 L) were collected concurrently from either the pond perimeter (SED) or from a benthic area under the water intake about 8 to 10 feet from the surface (SED_B), using benthic dredge (WILDCO® Fieldmaster® Mighty Grab II Dredge). Collection vessels were sanitized with 10% bleach and rinsed with sterile water between samplings. All samples were stored on ice in coolers in the field and transported to lab for refrigeration until analyses were conducted within 24 hours of collection. Aliquots of water and sediment samples were used to inoculate enrichment cultures for microbial as described below.

Analysis of performance of detection methods. As previously described (Wright CPS Final Report, 2013) and summarized in Figure 1 in the appendix, *Salmonella* was enumerated by a MPN protocol using three dilutions of triplicate enrichment cultures, with some small modifications of our previous protocols (Rajabi et al., 2011). We conducted statistical evaluation of methods used in prior studies in order to validate an alternative protocol for *Salmonella* detection and enumeration. Basically, aliquots of 500ml, 100ml, and 10ml of water or 100g, 10g, and 1g of sediment (sediment was allowed to settle and water decanted) were inoculated into equal volumes of double strength (2×) lactose broth (Fisher Scientific Inc.), except 1g of sediment was inoculated into 10 ml of 1x lactose broth. Inoculated lactose broth cultures were incubated at 37°C for 24 hours. One milliliter of lactose broth was transferred to 9ml of tetrathionate (TT) broth (Fisher Scientific Inc.) with 20ml/L of iodine potassium solution (Sigma-Aldrich) for 24 hours at 37°C. A total of 147 water samples and 147 sediment samples were examined, and thus, there were a total of 294 MPN samples (i.e., 2646 TT cultures from a 3 tube x 3 dilution MPN). Type strain *S. enterica* Typhimurium LT2 (ABC Research Laboratories, Gainesville, FL) was used as a positive control for enrichment cultures and PCR analysis. Inoculated lactose broth cultures were incubated at 37°C for 24 hours. One milliliter of lactose broth was transferred to 9ml of TT broth (Fisher Scientific Inc.) with 20ml/L of iodine potassium solution (Sigma-Aldrich) for 24 hours at 37°C. Following enrichment, presumptive *Salmonella* colonies were isolated on selective and differential agars, Xylose-Lysine-Tergitol 4 (XLT4, Remel, USA) and CHROMagar Salmonella Plus (CSP, CHROMagar Microbiology, Paris, France). Typical colonies were subsequently confirmed by PCR (Stone et al., 1994), using nucleotide sequences 5'-TGCCTACAAGCATGAAATGG-3' (*invE*) and 5'-AAACTGGACCACGGTGACAA-3' (*invA*). DNA extract (1µl) was mixed with 2.5 µl of 10× buffer (5 PRIME), 400 nM of each deoxynucleotide triphosphate (dNTP, Invitrogen), 400 nM of each primer (Sigma-Aldrich), and 0.5 µl of *taq* polymerase (5 PRIME) in a final volume of 25 µl. The PCR conditions included an initial denaturation at 94°C for 5 min, followed by 30 cycles of DNA amplification under the following conditions: 94°C for 30s, 58°C for 30s, and 72°C for 30s with a final extension at 72°C for 5 min. Confirmed positive strains from MPN analysis were spread on LuB agar plates, incubated overnight, and harvested for frozen storage (-80°C) in LuB with 50% glycerol for phenotypic and genotypic analyses.

The efficacy of six different downstream detection methods for confirmation of *Salmonella* in the TT broth from the MPN enrichment protocol described above was examined. Specifically, these detection methods are as follows: (1) XLT4-CSP-PCR method was considered the gold standard, and colonies were isolated from TT broth on two selective agars, XLT4 and CSP, followed by PCR for individual isolated colony confirmation from one or both agars; (2) TT-PCR was an attempt at more rapid detection using PCR of the DNA extracted directly from TT broth without colony isolation; (3) XLT4-CSP used the criteria for *Salmonella* positive samples based on isolation of typical colonies on either XLT4 and/or CSP agar but without PCR confirmation; (4) XLT4-PCR only considered XLT4 presumptive positive colonies followed by PCR confirmation for individual colony; (5) XLT4 used XLT4 but without PCR confirmation for individual colonies; (6) CSP-PCR used isolation on CSP followed by PCR for individual colony confirmation; and (7) CSP used CSP without PCR confirmation.

As described in previous studies (Carrique-Mas et al., 2009, Hyeon et al., 2012, Soria et al., 2012), a variety of performance criteria are usually calculated to describe the performance of binary detection methods. These performance measures are defined as follows: (1) sensitivity (the true positive rate) is the (conditional) probability to classify a unit as positive given that the unit is positive, (2) specificity (the true negative rate) is the (conditional) probability to classify a unit as negative given that the unit is negative, (3) positive predictive value (the positive precision rate) is the (conditional) probability that the unit is positive given that it is classified as positive, and (4) negative predictive value (the negative precision rate) is the (conditional) probability that the unit is negative given that it is classified as negative. Often, other performance measures are also reported, including false positive rate (1-sensitivity), false negative rate (1-specificity), false discovery rate (1-positive predictive value), and false acceptance rate (1-negative predictive value). In order to calculate these measures, a “gold standard” is needed as a baseline or common basis of comparison, which would ideally be an infallible detection method. The XLT4-CSP-PCR method is a commonly used and scientifically sound method and was considered as the gold standard in these analyses. The XLT4-CSP-PCR method classifies a unit (i.e., a TT sample) as positive if it contains colonies with typical *Salmonella* appearance on either agar and these isolate(s) are later confirmed as *Salmonella* positive using PCR; otherwise the unit is classified as negative. The estimates of these performance measures are usually based on the *confusion matrix* of actual and predicted group membership of the classified units which has the following values (entries): TP is the number of true positive units (in our case, the number of XLT4-CSP-PCR positive units that were classified as positive by the detection test), TN is the number of true negative units, FP is the number of false positive units, and FN is the number of false negative units. The estimates of the performance measures are calculated as follows: sensitivity = TP / (TP + FN), specificity = TN / (TN + FP), positive predictive value = TP / (TP+FP), and negative predictive value = TN / (TN + FN).

To measure the agreement between the other assays and the XLT4-CSP-PCR method, we calculated Cohen’s kappa coefficient (Petersen and Wachmann, 1998). The kappa coefficient is given by $\kappa = \frac{\Pr(A) - \Pr(E)}{1 - \Pr(E)}$, where $\Pr(A)$ is the probability that the two detection tests agree

with each other and $\Pr(E)$ is the probability that the two detection tests agree by chance. The kappa coefficient takes values between 0 and 1, and higher values of kappa show stronger agreement between the two methods. Note that the agreement between two detection tests is calculated irrespective of whether one of the two tests is a gold standard. When we interpret the kappa values, we adopt the same interpretation as (Soria, Soria and Bueno, 2012). Specifically, a kappa value between 0.80 and 1.00 shows excellent agreement, between 0.61 and 0.80 shows good agreement, between 0.41 to 0.60 shows fair agreement, between 0.21 to 0.4 shows slight agreement, while a kappa value between 0.01 to 0.20 shows poor agreement. In addition, we also perform McNemar’s test of homogeneity of positive detection rates of the two methods. The

McNemar’s test is given by $X^2 = \frac{(b - c)^2}{b + c}$, where b and c are the number of discordant results of

the two detection tests. Specifically, b is the number of samples classified as positive by the first method and negative by the second method and c is the number of samples classified as negative by the first method and classified as positive by the second method. The reference distribution of McNemar’s test is the chi-squared distribution with one degree of freedom. All statistical

analyses were performed in R (R core Team, 2013). We used the contributed R package *bdpv* (Schaarschmidt, 2012) to estimate and to construct confidence intervals for the performance measures, and the contributed package *irr* (Gamer et al., 2012) to compute the kappa coefficient and to test its significance. McNemar's test is available in the *stat* package of the R statistical language.

Analysis of performance of the cross-streaking method. The cross-streaking method is a novel detection method that was developed as a consequence of preparing the strain collection for genetic analyses, and is proposed as an alternative to the application of PCR for confirmation of colony identification. Samples from TT broth were initially streaked to XLT4, and presumptive positive colonies were subsequently re-streaked to CPS agar. The cross-streaking method classifies a colony as positive if it exhibits typical *Salmonella* appearance on both XLT4 and CSP agars and it classifies a colony as negative if it does not. In order to ensure isolation of a single colony from primary XLT4 agar isolation, colonies were also re-streaked to XLT4. The PCR assay was considered to be the gold standard when we calculated the performance measures of the cross-streaking method. In our statistical analysis, we compared the results for bacterial isolates exhibiting presumptive positive colonies on at least one selective agar and confirmed as *Salmonella* positive by PCR in this study (n=1349) and also 30 isolates that were confirmed positive *Salmonella* strains from other aquatic environmental sources (Rajabi et al., 2011). The remaining 261 colonies were non-*Salmonella* isolates as determined by PCR. As described above, sensitivity, specificity, positive predictive value, and negative predictive value were calculated to evaluate the performance of the cross-streaking method. The strength of agreement was again calculated using the kappa coefficient and McNemar's test of agreement was performed to test the equality of the positive detection rates of the cross-streaking and PCR detection methods. Material cost was calculated based on precise reagent consumption including lactose broth, tetrathionate broth, XLT4 agar, CSP agar, PCR reagents, gel electrophoresis reagents, and approximate costs of other disposable materials, such as conical tubes, wood applicators, 1.5 ml centrifuge tubes, pipette tips Total expenses per MPN for each detection method was calculated in US dollars.

Antibiotic resistance. Strains were screened for antibiotic resistance using susceptibility test discs according to the manufacturer's protocol (BBL™ Sensi-Disc™). Antimicrobial MICs for *Salmonella* isolates were determined by the CDS test standard (Bell et al., 2009). The following antibiotics were tested: 30 µg amikacin, amoxicillin-clavulanic acid, 25 µg ampicillin, 30 µg cefoxitin, 5 µg ceftriaxone, cephalothin, 30 µg chloramphenicol, 2.5 µg ciprofloxacin, 10 µg gentamicin, 10 µg imipenem, 50 µg kanamycin, 30 µg nalidixic acid, 25 µg streptomycin, sulfamethoxazole-trimethoprim and 10 µg tetracycline. In details, *Salmonella* were streaked for isolation on tryptic soy agar (TSA, Difco, Becton Dickinson) and incubate overnight at 37°C. One or two overnight colonies from TSA) were stabbed to 1 ml of sterile saline (0.85% NaCl) to match the turbidity to a 0.5 McFarland Standard. Shake the tube and then dip a swab in the inoculum and spread the swab 3 times onto Sensitest agar (Oxoid), rotating 45° each time, and dry 5-10 min (max 30 min). Apply no more than 5 equidistant antibiotic discs per plate by dispensing four from a disc dispenser, the 5th antibiotic disc will be placed with a sterile tweezer in the center. Measure zones of inhibition after incubation at 35-37°C for 18 h and classify as susceptible according to the antibiotic's chart for enterobacteriaceae (Bell et al., 2009).

Molecular typing and virulence potential. Molecular typing was conducted using frozen stocks of PCR-confirmed strains from studies described above. The DiversiLab rep-PCR system for Salmonella (BioMerieux) was used to determine similarity among strains in this study relative to reference strains from both clinical and environmental sources. PCR was conducted according to manufacturer's instructions and products were evaluated using capillary electrophoretic analysis for automated comparison to an online database of >300 strains (Rajabi et al., 2011). PFGE analysis was conducted using bacterial isolates suspended in cold buffer containing 1 M NaCl, 10 mM Tris pH 8, and 10 mM EDTA for DNA isolation. DNA was digested in enzyme buffer with restriction enzyme XbaI. After digestion, DNA was loaded into wells in agarose gel. The agarose gel was placed into the Electrophoresis Cell (Bio Rad) to run electrophoresis. After electrophoresis, the gel were stained with ethidium bromide, the image was captured by Gel/Chemi Doc and saved in computer as TIFF files using software Quantity I (Bionumerics). Images were analyzed and the similarity among different strains was characterized using computer software GelCompar II (Applied Maths). Whole genome sequencing was conducted using FDA protocol for MiSeq (Illumina). Virulence potential was evaluated by comparison of molecular typing and WGS results of strains from environmental vs. clinical sources.

RESULTS

Development of cross-streaking method for enumeration and confirmation of *Salmonella*.

Table 1 shows the confusion matrix of TT_PCR compared with XLT4-CSP-PCR (n=1602). These results indicated that the XLT4-CSP-PCR method had a higher positive detection rate (6.1%) than TT_PCR (3.0%). Results were confirmed by the McNemar's test, which is highly significant ($X^2=37.96$, $P<0.0001$). Furthermore, Cohen's kappa statistic is 0.47, which shows these two detection methods are only in a fair agreement. Evaluation of the performance of TT_PCR also showed that TT_PCR attained high specificity (0.99) and high negative predictive value (0.96) but had a low sensitivity (0.35) and a low positive predictive value (0.78). These data showed that pre-screening of MPN enrichment samples by TT_PCR did not yield results that were consistent with XLT4-CSP-PCR. Overall, TT_PCR is two times faster, less labor intensive, and less expensive (see estimate below) than XLT4-CSP-PCR; however, its sensitivity (of 0.35) is small compared to XLT4-CSP-PCR. Moreover, *Salmonella* strains are not recovered using the TT_PCR test and are not available for further molecular characterization. Decreased positive detection rate of TT-PCR compared to XLT4-CSP-PCR may result from inhibition of PCR by compounds in TT DNA extract. The performance of TT_PCR might be increased by using more refined DNA extraction methods (Klerks et al., 2006) or by using more sensitive PCR assays (e.g., with lower detection limits such as nested PCR) (Klerks et al., 2004). Considering the higher positive detection rates and the availability for further molecular typing for isolated strains, we consider XLT4-CSP-PCR as a better method in this circumstance.

Evaluation of TT broth samples (n=2646) from various water and sediment sources showed differences in performance of XLT4 and CSP as initial isolation agars. Using isolation on two selective agars followed by PCR confirmation (XLT4-CSP-PCR method) as the gold standard, the performance measures for using both agars without PCR (XLT4-CSP) or for using XLT4 vs. CSP agar alone with (XLT4-PCR, CSP-PCR) or without PCR confirmation (XLT4, CSP) were calculated in terms of their sensitivity, specificity, positive predictive value, and negative predictive value. Although XLT4-CSP, XLT4, and CSP had high sensitivities and high negative predictive values, they were not considered ideal methods because the positive predictive values

were all quite low (not shown). Using only one selective agar for initial isolation as significantly different from XLT4-CSP-PCR, although the values of sensitivity, specificity, positive predictive value, and negative predictive value were all within a good range. The efficiency of the agars without assuming that the XLT4-CSP-PCR is the gold standard was also assessed (not shown). XLT4-PCR had the highest agar efficiency rate, lowest cost (described below), and lowest labor intensity due to fewer false positive results. Also, it was noted that an improved strategy might be to identify presumptive positive colonies on XLT4 plates at 20 rather than 24 hours of incubation, as suggested by the manufacturer's protocol, since an increase in the false positive rate for colonies after 20 hours of incubation was observed (data not shown).

Based on evaluation of experimental methods described above, a **cross-streaking method** was developed and represents a new discovery. Sequential isolation (cross-streaking) of typical colonies from the primary isolation agar (XLT4) to the alternate secondary agar (CSP) was highly predictive for colony identification by PCR. XLT4 was selected over CSP for primary selection based on relative performance and cost of the agars. All isolates from that were positive on both agars were also confirmed as *Salmonella* positive by PCR (n=1611). Only one isolate from a prior study of the Suwannee River (Rajabi et al., 2011) was found to be PCR positive, CSP positive, but negative on XLT4. The cross-streaking method was highly performant: sensitivity=0.99, specificity=1.00, PPV=1.00, and NPV=0.99. The kappa coefficient of agreement with PCR assay was 0.99. Cross-streaking was a more cost-effective (Table 2) at \$11.74/MPN (37% less than gold standard) and showed performance standards nearly equivalent to the gold standard (Table 2). Further studies are required to identify potential false positive and false negative results using this assay.

Antibiotic resistance of wildlife vs. pond isolates.

A total of 40 strains collected from wildlife during 2011 were compared to strains collected from sampling pond water and sediments (n=130) as described above. Most strains were resistant to streptomycin for both groups (76%). Strains with multiple resistant (8%) were generally resistant to streptomycin and one other antibiotic (Ampicillin, Nalidixic Acid, Trimethoprim/ Cefoxitin Kanamycine), and were distributed evenly among wildlife vs. pond strains. Only two strains were resistant to 3 antibiotics: one from a turtle (Cephalothin, Kanamycine, Streptomycin) and one from a water sample (Ampicillin, Nalidixic Acid, and streptomycin).

Molecular typing.

PFGE analysis has been completed for a total of 380 strains of *Salmonella* either as part of this study or from prior research. Strains from wildlife (n=150) were obtained from 5 ponds (BB, MD2, CC2, SC, AND NP), while strains (n=148) from sediment and water were derived from all 10 ponds. Strains (n=30) from the Suwannee River from a previous study and strains (n=52) obtained from produce trace-back studies by the Florida Department of Agriculture were also PFGE typed. DiversiLab rep-PCR has been completed on a total of 380 strains. Including strains from wildlife (n=70), pond water or sediment (n=280), and from the Suwannee River (n=30). Whole genome sequencing (WGS) has been completed on 248 strains, including those from wildlife, pond water and sediment, the Suwannee River, and strains obtained from produce trace-back studies by the Florida Department of Agriculture, as well as isolates obtained from the Emerging Pathogens Institute from various sources.

Comparative analysis for PFGE and rep-PCR showed a total of 83 PFGE types and 16 rep-PCR types that were identified from alignments for a subset of strains from pond water and sediments (n=182). Greater diversity observed by PFGE compared to rep-PCR was based on differences in the level of discrimination and the number of bands generated by each assay. Clustering for rep-PCR used 85% similarity in banding patterns, while PFGE only grouped identical patterns. Thus, most strains within a single rep-PCR cluster showed multiple PFGE types. There was some agreement observed between the two methods, and most strains (59%) that clustered by one assay were also grouped by the other. Future alignments will adjust these parameters in order to improve these comparisons. Five strains that were untypeable by PFGE were discriminated by rep-PCR. Based on PFGE strains did not cluster by pond or season. Interestingly, Many genotypes (20%) from wildlife strains were unique for a single sample, and were not recovered from other species or from water or sediment. Furthermore, multiple genotypes were observed from any particular host species, and some genotypes were less specific and contained clusters with different species and/or water samples. Interestingly, many genotypes (20%) from wildlife strains were unique for a single sample. Based on PFGE strains did not cluster by pond or season.

Assessing Virulence Potential. Virulence potential can be assessed by using molecular typing methods to examine the genetic similarity of environmental strains compared to those derived from clinical infection. Using rep-PCR, a prior study showed *Salmonella* strains isolated from the Suwannee River were a genetically distinct population from those strains that were commonly associated with human disease. Most Suwannee River strains clustered (<85% similarity) with each other and generally did not cluster with clinical strains (Rajabi et al., 2011). Conversely, the clinical strains generally clustered with each other and not with environmental isolates. We used this approach to estimate virulence potential of strains collected from water or sediment sources derived from the 10 ponds described above and showed that pond strains (50% of n=200) were more likely to group with strains from clinical origin and were designated as C type. Strains that clustered with strains from other environmental sources (mostly the Suwannee River) were designated as E-type and were much less frequent (35%) than C-type (Figure 2 in appendix). The remaining strains (15%) appeared to be unique to the database and only clustered with other pond strains. In contrast, strains from wildlife origin were predominantly E-type (87%) and only 10% of wildlife strains were C-type with the remaining strains unique (3%, Figure 3 in appendix).

To date, we have sequenced 248 *Salmonella* genomes. Preliminary phylogeny of a subset of WGS maximum likelihood analysis showed good (about 70%) agreement with rep-PCR (Figure 4 in appendix), and further WGS analysis is being conducted. In addition to contributing to the national WGS database of *Salmonella* isolates, we are using this opportunity to compare phylogenies based on WGS data with PFGE and other typing methods. We are exploring the demographic history of this pathogen in Florida and Georgia using a longitudinal sample of isolates. What is revealed is a complex dynamic of isolates collected from environmental and wildlife sources across north-central Florida in South Georgia. This technology promises to enhance our current national surveillance framework as well as expand our understanding of *Salmonella* ecology and transmission in Florida.

Outcomes and Accomplishments

One of the accomplishments of this research was to develop a novel “low tech” cost effective alternative to PCR for enumeration and identification of *Salmonella*. This was an unexpected outcome, which was a consequence of data analysis from prior CPS-funded research and the process of building the strain collection for genetic analysis. The cross-streaking method offers the specificity and sensitivity nearly equivalent to PCR without the requirement for specialized equipment or training. The method would allow for increased number of samples evaluated, and hence increase the limit of detection, which is always an issue for detection of pathogens in the environment.

Perhaps the most important outcome of this project was to link the genomic project with the Florida Department of Health. Recent comparison of different typing methods to whole genome sequencing has indicated that they lack of discrimination power of comparative genomics (Cao et al., 2013). PFGE was not able to discriminate highly clonal strains, and neither PFGE nor MLST correctly identified the relationship among lineages within different strains *Salmonella* Newport that were apparent by WGS. Evaluation of WGS for outbreak detection found SNP analysis outperformed PFGE, but noted that the validation of this approach requires additional evaluation of sequencing platforms analytical procedures, and larger databases (Leekitcharoenpho, 2014). As more genomes become available and methods become standardized, WGS should provide better understanding of the evolution and ecology of *Salmonella* subspecies and serotypes. With the advent of next generation sequencing platforms, high throughput is feasible, and genomic sequencing could provide the basis for the evolution of PulseNet into a similar network based on WGS, i.e. a “GenomeNet”, that would provide more rapid and much more accurate analysis of identifying strains associated with outbreak events.

Summary of Findings and Recommendations

- **We discovered a novel “cross-streaking” method that provides cost effective detection and enumeration of *Salmonella* from aquatic reservoirs with specificity and sensitivity comparable to PCR methods.**
- ***Salmonella* strains derived from wildlife from irrigation ponds in Georgia represent a genetically diverse population.**
- **There was no association of a particular genotype with a particular species, pond, or season.**
- **Although 50% *Salmonella* strains from irrigation pond water or sediment showed genetic similarity to strains from clinical origin, strains from wildlife were mostly (87%) clustered with strains from environmental sources.**

Unfortunately, the use of fecal contamination indicators frequently does not reflect distribution of foodborne pathogens such as *Salmonella enterica*, and ensuring food safety may require direct detection and enumeration of pathogens in agricultural settings. Herein, we summarize novel methods that may facilitate these efforts, and filtration combined with IMS may result in even greater sensitivity for evaluation of environmental samples. We recommend further study before the introduction of stringent measures to control wildlife access to irrigation water. Although we water and sediment samples frequently contained *Salmonella* with genetic similarity to clinical strains, in general, strains from amphibians and reptiles did not appear to serve as a reservoir for these strains. Currently, a consortium of FDA, CDC, and USDA researchers, as well as various

public health and academic institutions are building a whole genome sequence database that will eventually permit public access the literally thousands of strains from different geographic locations, food product, agricultural, veterinary, and clinical sources. The prospect of WGS for a “GenomeNet” approach not only hold promise from more rapid and accurate source tracking during outbreak, but will potentially help to define virulence potential of the wide repertoire of diverse *Salomonella* populations for more informed and science-based policy decisions and management strategies.

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APPENDICES

Publications and Presentations (required)

Publications

1. Luo, Z., G. Gu, C. G. Mihai, P. Adams, G. Vellidis, A. H. C. van Bruggen, **A. C. Wright**. Development of a novel cross-streaking method for isolation, confirmation, and enumeration of *Salmonella* from irrigation ponds. *J. Microbiol. Methods*. In Press. The manuscript has been assigned the control number AEM00954-14.
2. Luo, Z., G. Gu, P. Adams, G. Velidis, A. H. C. VanBruggen, M. Danyluk, **A. C. Wright**. Distribution and genetic diversity of *Salmonella enterica* isolated from Irrigation Water in the Suwannee River Watershed. (In preparation to *Appl. Environ. Microbiol.*)
3. Li, B. Vellidis, G. H., Liu, M. Jay-Russell, S. Zhao, Z Hu, A.C Wright, and C. Elkins. Improved Detection and Isolation Scheme Reveals Diversity and Persistence of *Salmonella enterica* Subtypes in Surface Water in Southeastern U.S. (under review by *Appl Environ. Microbiol.*)
4. Gu, G., Luo, Z., Cevallos-Cevallos, J., Adams, P., Vellidis, G., Wright, A., and van Bruggen, A. 2013a. Factors affecting the occurrence of *Escherichia coli* O157 contamination in irrigation ponds on produce farms in the Suwannee River Watershed. *Canadian Journal of Microbiology*, 59(3): 175-82.
5. Gu, G., Luo, Z., Cevallos-Cevallos, J., Adams, P., Vellidis, G., Wright, A., and van Bruggen, A. 2013. Occurrence and population density of *Campylobacter jejuni* in irrigation ponds on produce farms in the Suwannee River Watershed. *Canadian Journal of Microbiology*, 59(5): 339-46.

Presentations

1. **Li, B.**, J.-Q. Chen, M. Jay-Russell (WCFS), G. Vellidis (UGA), A. Wright (UF), Z. Hu, J. Gangiredla, S.A. Jackson, and C.A. Elkins (2013). Rapid Detection and Serovar Identification of *Salmonella* by Real-Time PCR and Microarray from Environmental Irrigation Water Sources. Poster presentation at the American Society for Microbiology. Denver, CO
2. Luo, Z., G.Gu, P. Adams, G. Velidis, A. VanBruggen, M. Danyluk and A.C. Wright. (2013) Distribution and Characterization of *Salmonella enterica* from Irrigation Water in the Suwannee River Watershed. Poster presentation at the International Association of Food Protection Annual Meeting.
3. Luo, Z., G.Gu, P. Adams, G. Velidis, A. VanBruggen, M. Danyluk and A.C. Wright. (2013) Science-based evaluation of regional risks for *Salmonella* contamination of irrigation water at mixed produce farms in the Suwannee River Watershed. Poster presentation at the Center for Produce Safety Annual Meeting. Rochester, N.Y.
4. Luo, Z., G.Gu, P. Adams, G. Velidis, A. VanBruggen, M. Danyluk and A.C. Wright. (2013) Science-based evaluation of risks associated with wildlife exposure for contamination of irrigation water by *Salmonella*. Rochester, N.Y.
5. Waterman, K., T. Azarian, Z. Luo, M. Allard, A. C. Wright, J. Johnson, M. Salemi (2014) Whole-Genome Sequencing for National Surveillance of Enteric Diseases. UF EPI Research Day.

Budget Summary (required)

Budget Category	Total Budget	Spent	Committed	Projected	Balance	Health
All Payroll		\$53,504.00	\$34,472.05	\$0.00	\$0.00	\$0.00
Other Expense		\$688.93	\$688.93	\$0.00	\$0.00	\$0.00
Materials & Supplies		\$23,039.89	\$23,039.89	\$174.67	\$0.00	(\$174.67)
Tuition		\$8,077.14	\$8,077.14	\$0.00	\$0.00	\$0.00
Domestic Travel		\$9,694.99	\$1,687.72	\$0.00	\$0.00	\$8,007.27
Direct Total		\$75,973.00	\$67,965.73	\$174.67	\$0.00	\$7,832.60
Indirect Total F&A		\$1,723.60	\$1,723.59	\$0.00	\$0.00	\$0.01
Project Total		\$77,696.60	\$69,689.32	\$174.67	\$0.00	\$7,832.61

Remaining grant balance was because one of the participants changed from a full-time employee with benefits to a graduate student with stipend that were greatly reduced from the original package. She also received a partial assistantship from the department that further reduced that grant expenditure. Additional part-time students were hired but without benefits and hence these funds were not spent. This funding expires 6/30/2014. We anticipate an additional \$2000 in travel to CPS meeting and \$4000 in publication costs for a final balance between \$1832.

Tables and Figures (optional)

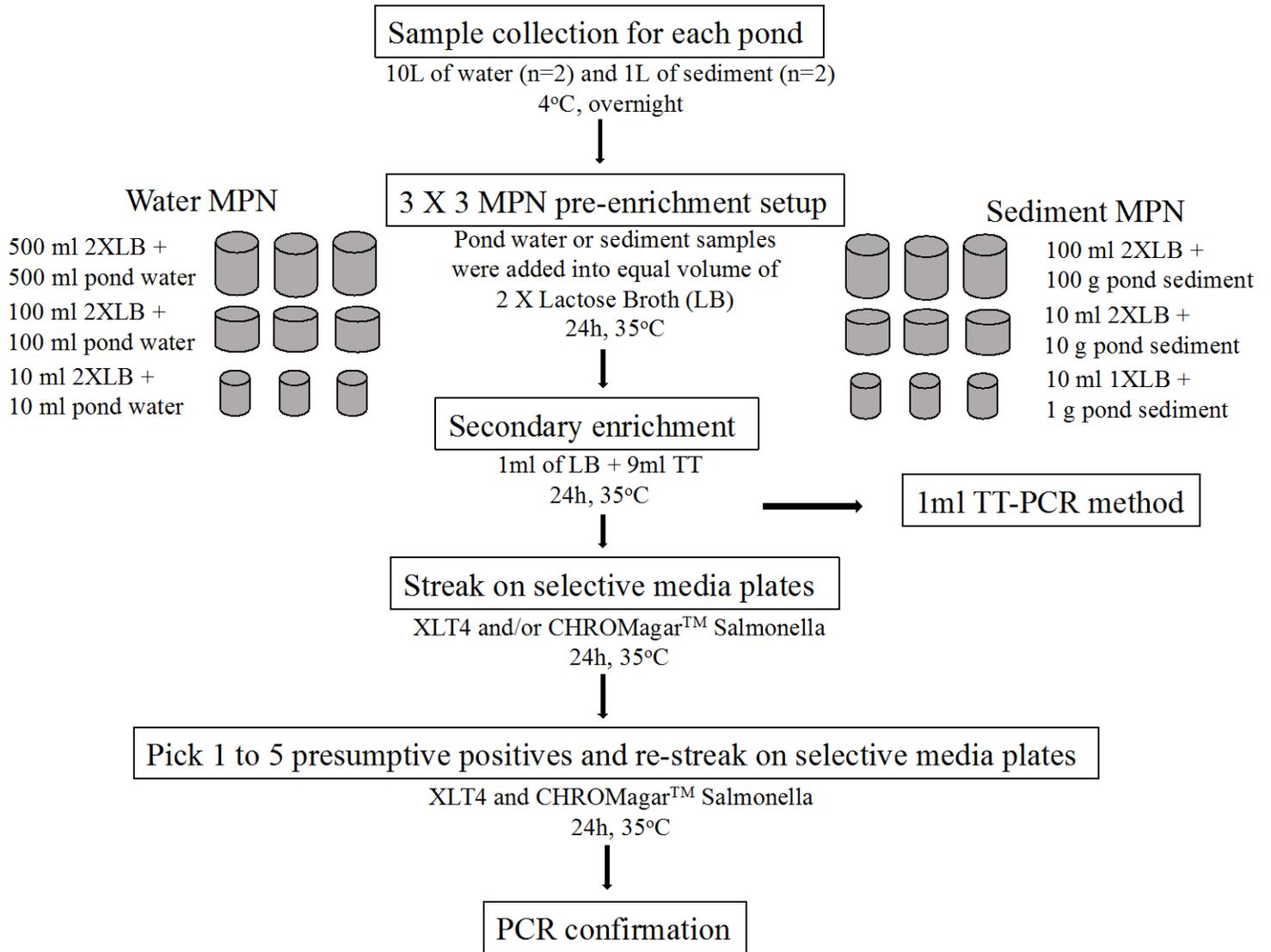


Figure 1. Flowchart diagram for most probable number (MPN) enumeration, isolation and identification of *Salmonella*. TT = tetrathionate broth; XLT4 = xylose lysine desoxycholate agar with tergitol 4; LB= Lactose Broth.

Table 1. Confusion matrix for XLT4-CSP-PCR vs. TT-PCR (n=1602 TT tubes) for confirmation of *Salmonella* in TT broth (n=1602 tubes)

	XLT4-CSP-PCR positive	XLT4-CSP-PCR negative
TT_PCR positive	35	10
TT_PCR negative	64	1493

Table 2. Assessment of material cost per MPN for different *Salmonella* detection assays.

Detection Method ^a	Material Cost (USD) per MPN ^b						Total cost	Sensitivity ^d	Specificity ^d
	LB	TT ^a	XLT4	CSP	PCR	Other ^c			
XLT4-CSP-PCR	3.15	1.88	2.80	5.60	2.64	2.50	18.60		
TT-PCR	3.15	1.88	0	0	5.94	2.50	13.50	0.35 (0.26, 0.45)	0.99 (0.99, 1.00)
XLT4-CSP	3.15	1.88	2.80	5.60	0	2.50	16.00	1.00 (0.98, 1.00)	0.85 (0.83, 0.86)
XLT4-PCR	3.15	1.88	2.80	0	2.64	2.50	13.00	0.95 (0.92, 0.98)	1.00 (1.00, 1.00)
XLT4	3.15	1.88	2.80	0	0	2.50	10.30	0.95 (0.91, 0.98)	0.93 (0.92, 0.94)
CSP-PCR	3.15	1.88	0	5.60	2.64	2.50	15.08	0.80 (0.74, 0.85)	1.00 (1.00, 1.00)
CSP	3.15	1.88	0	5.60	0	2.50	13.10	0.80 (0.75, 0.85)	0.90 (0.89, 0.91)
Cross-streaking	3.15	1.88	2.80	1.40	0	2.5	11.73	0.999 (0.996, 1.000)	1.000 (0.986, 1.000)

a) Detection methods are described in text and Figure 1.

b) Materials include lactose broth (LB), tetrathionate broth (TT), XLT4 and CSP = CHROMagar *Salmonella* Plus agars, and PCR reagents (including electrophoresis reagents) based on 9 reactions/MPN for TT-PCR and 4 reactions for the other assays. Agar costs are based on streaking ½ plates for initial isolation from TT broth and 1/8 plates for restreaking of colonies in the cross-streaking assay.

c) Other materials include wood applicators, conical tubes, microcentrifuge tubes, pipette tips and petri dishes.

d) Performance measures are described in text and use the XLT4-CSP-PCR method as gold standard

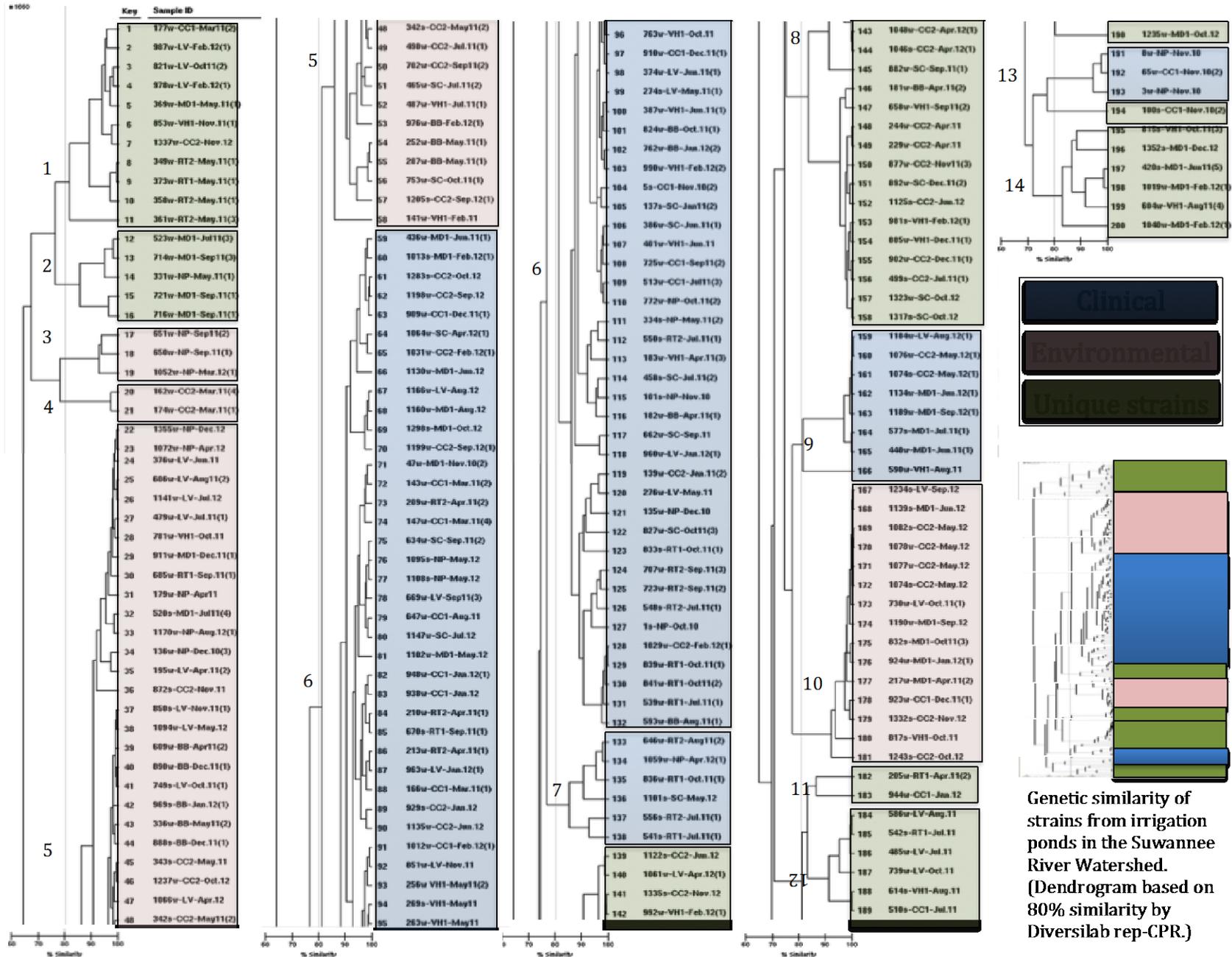


Figure 2. DiversiLab rep PCR Molecular typing of *Salmonella* from pond water and sediments. Strains (n=200) from ponds in this study are shown in the dendrogram above, and similarity (>80%) of these strains to strains from clinical infection (50%) or from other environmental sources (35%) were determined by dendrogram of all strains examined by rep-PCR (not shown).

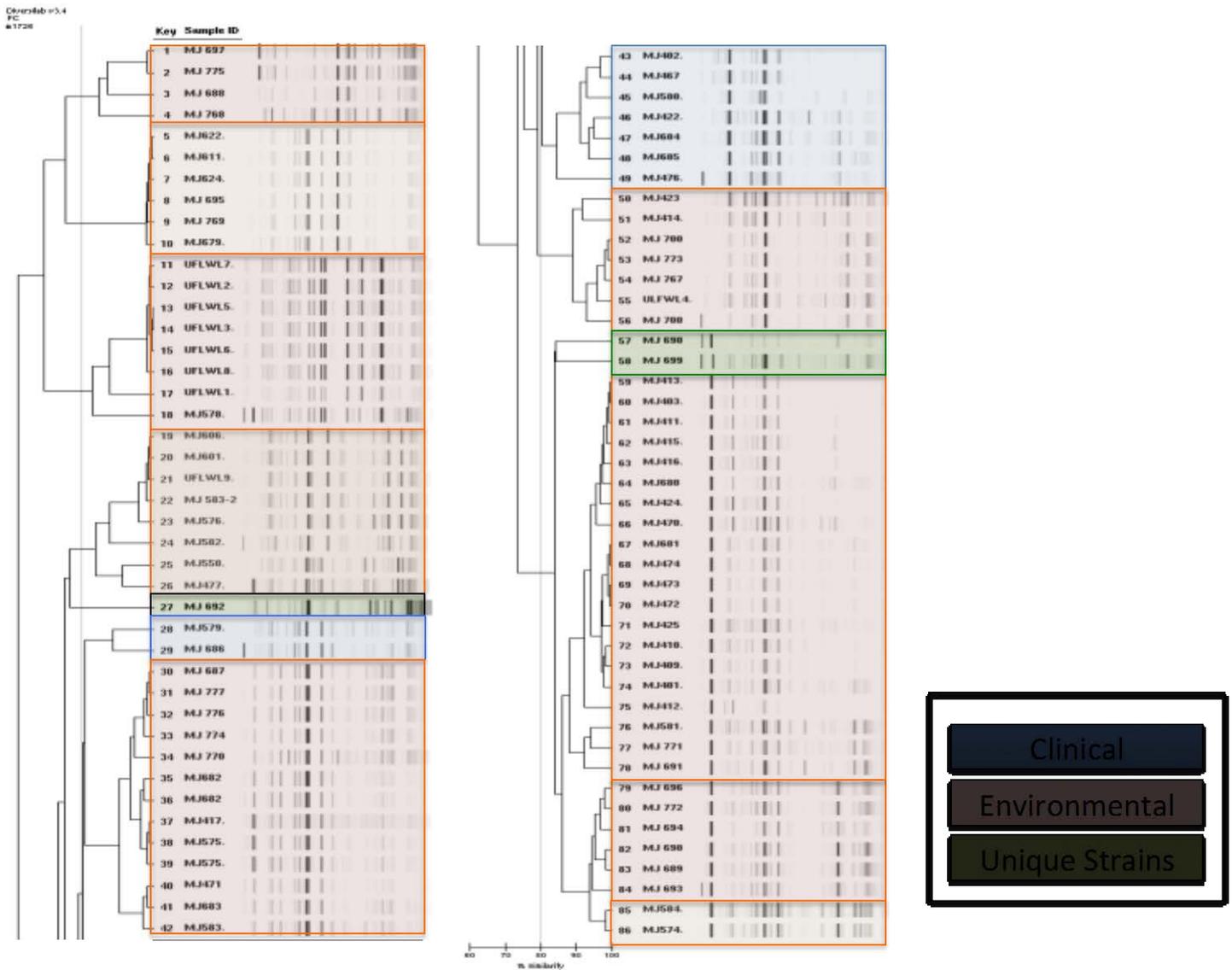


Figure 3. DiversiLab rep PCR Molecular typing of *Salmonella* from wildlife. Strains (n=86) from wildlife in this study are shown in the dendrogram above, and similarity (>80%) of these strains to strains from clinical infection (10%) or from other environmental sources (87%) were determined by dendrogram of all strains examined by rep-PCR (not shown).

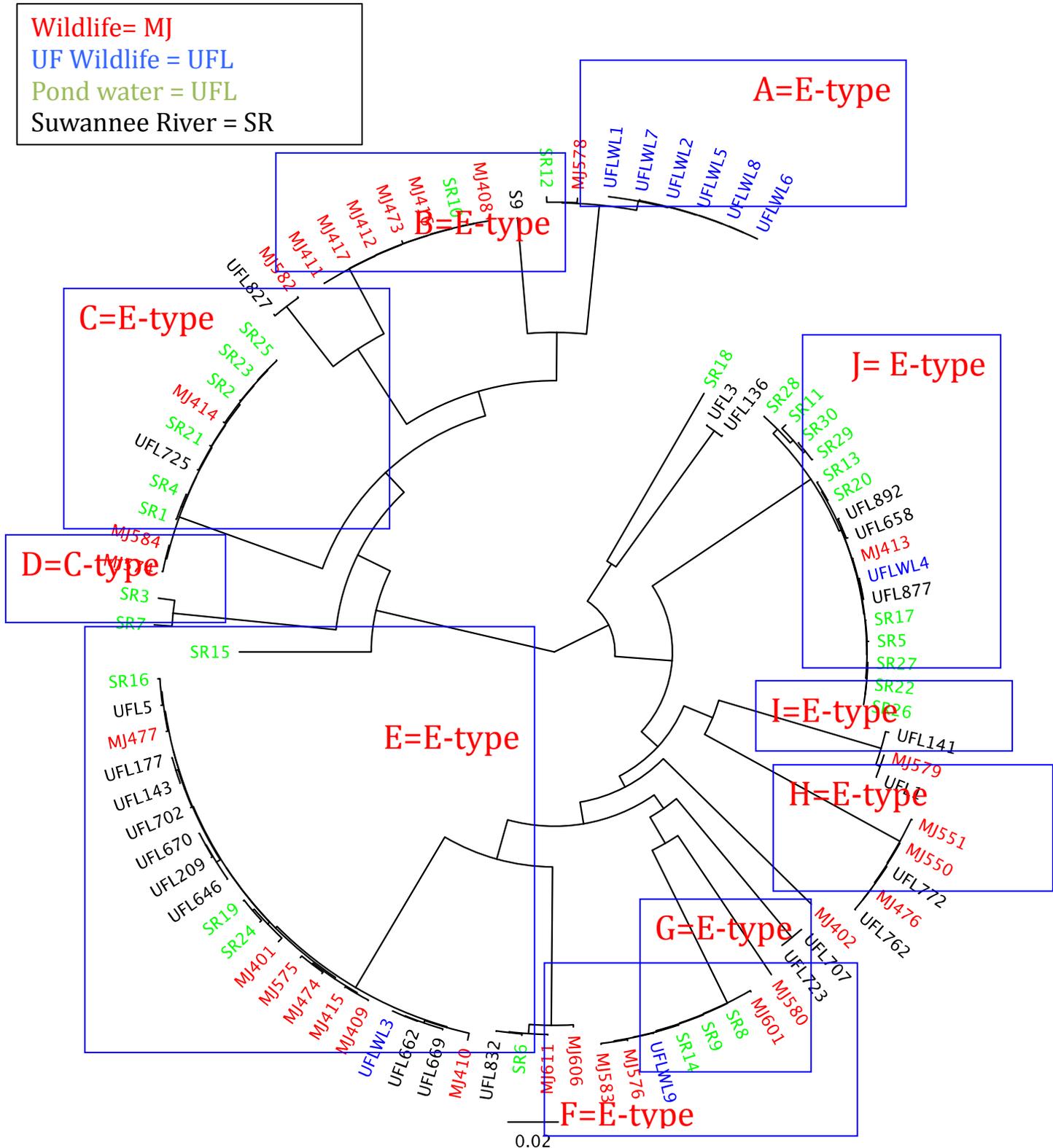


Figure 4. Comparison of Whole Genome Sequencing (WGS) to rep-PCR molecular typing. WGS was evaluated using Maximum Likelihood Analysis of single nucleotide polymorphisms (SNPs). Clusters (A-J) are based on SNP analysis of WGS. Clinical-type (C-type) and environmental type (E-type) are based on the rep-PCR similarities described in Figures 2 and 3 above.