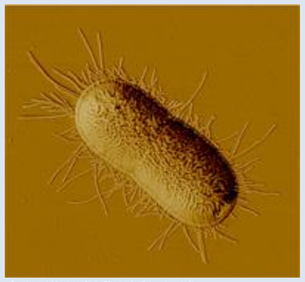
Salmonella spp. numbers much greater than indicator bacteria in environmental waters

Timothy M. Smith, Sarah F. Lucento, David W. Buckalew
Department of Biological and Environmental Sciences
Longwood University
Farmville, VA 23909



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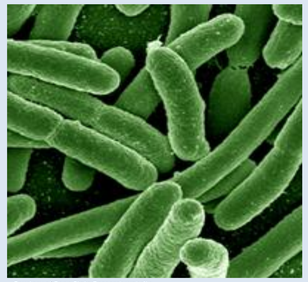

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Introduction

Since the acceptance of the Germ Theory of Disease, society has been interested in water quality and its influence on public health. Awareness of causal links to contaminated drinking water led to questions about the best gauge or indicators of water. As early as 1880, Von Fritsch suggested the use of *Klebsiella pneumoniae* and *K. rhinoscleromatis* as suitable indicators due to their presence in human feces (Geldreich, 1978). Five years later, Theodor Escherich discovered *Bacillus coli* (now *Escherichia coli*) whose presence was also observed in high numbers in the feces of warm blooded animals (Escherich, 1885). Several workers at that time argued for the use of **total coliform** as the gauge of fecal contamination in water (Hutchinson and Ridgway 1977). However, in 1905, Alfred MacConkey proclaimed that many coliforms in water were not of fecal origins (MacConkey, 1905). This led scientists to focus their attention mainly on *E. coli* while using total coliforms as a very loose guideline, which, even over a century later, is still the focus of current water quality assays.

Water quality is of as much interest now as it was back then when Pasteur and Koch were helping postulate the Germ Theory of Disease. Since then, there have been many discoveries of pathogens which can be transmitted via the fecal-oral route. Some of these pathogens include bacterial species such as: *E. coli* O157:H7, *Campylobacter jejuni, Salmonella enterica,* enteric viruses, and certain protozoans, to name a few -- all of which cause some form of gastroenteritis which, in some cases, can been fatal. This understanding has increased both the interest and the need to ensure safe water quality for human contact.

The question posed by this study is: How effective are *E. coli* counts in predicting the counts of potential pathogens, in this case, specifically *Salmonella species,* in local streams in south-central Virginia? Recent reports from the literature show mixed results. Some studies have shown little to no correlation of *Salmonella spp.* to indicator bacteria (Polo et al., 1998) while others have shown a significant correlation between *Salmonella spp.* and indicator bacteria (Efstratiou et al., 1998).

Materials and Methods

Salmonella Isolation and Enumeration

Water samples were collected from three locations: Appomattox River (APP2), Saylor's Creek (SAY5), and Green Creek (GRE16). The samples were collected by lowering a sterile container mid-column into the streams, avoiding the uptake of autochthonous debris. Samples were then placed on ice and transported back to the laboratory for processing. Two field duplicates were taken during each collection event. Samples were usually processed within two hours of collection.

In the laboratory, the samples were assayed via membrane filtration. One mL of sample was diluted with sterile, buffered water and filtered through 0.45 um pore size filter membrane (Millipore, Bedford, MA) and transferred to 50 mm petri plates containing 1.5 mL of Tetrathionate enrichment broth enhanced with novobiocin (40 mg/L) and incubated at 35^{o}C for 6-8 hours. The samples were then transferred to 50 mm petri plates containing 1.5 mL of sterile Brilliant Green Bile broth and incubated an additional 24 hrs at 35^{o}C.

The plates were enumerated by counting all colony forming units (CFU) that were thought to be *Salmonella spp.* based upon colonial phenotype (i.e. color and morphology). This was usually all of them except for those appearing as yellow. Any CFU with a hint of pink, orange, or red was counted as positive. Many of the representative colonies were transferred to Triple Sugar Iron (TSI) agar slants for confirmatory testing.

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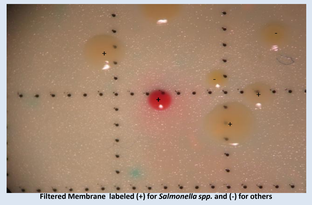


Filtered Membrane labeled (+) for *Salmonella spp.* and (-) for others

Coliform and E. coli enumeration

The same test water samples were split for the assessment of total coliform and *E. coli* via Defined Substrate Test using the Colilert (Idexx, Westbrook, ME) Quanti-tray 2000 system. Twenty-five mL of water sample was diluted with 75 mL of sterile, buffered water and then processed based on the manufacturer's instructions and incubated at the same conditions as the filter membranes.

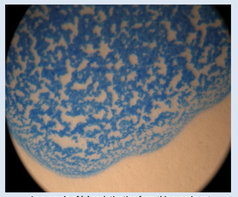
After incubation, the numbers of wells presenting chromogenicity were counted for total coliform enumeration. The trays were then subjected to UV light (365 nm) so as to count the fluorescing wells for *E. coli* enumeration. The results were compared to a most probable number based system with a quantification range of <1 to 9,680 CFU per 100 mL when using a 25 mL sample dilution. According to Edberg et al. (1990), confirmatory testing of coliform bacteria and *E. coli* using Colilert media is not necessary.

Salmonella spp. Confirmatory Tests

After each enumeration event, representative CFUs of different phenotypes, as well as CFUs from each different plate, were aseptically picked and transferred to a TSI agar slant and subsequently incubated at 35^{o}C for 48 hrs. After the incubation period, the acid reaction, CO_2 production, H_2S production, and the growth morphology were recorded. Tubes sharing identical morphologies, as well as TSI results, were grouped together and one tube was chosen at random for further confirmation using the Oxoid Rapid Salmonella Antibody Beads Test™.

All tubes that were unique, along with the representative tubes that shared colonial identity, were subjected to the Oxoid Rapid Salmonella Antibody Beads Test™. After mixing a loopful of suspect bacteria in the latex solution for ten seconds, the card was then tilted back and forth for an additional minute or so. Agglutination of the beads would signify a positive test for *Salmonella spp.*

Courtesy of Oxoid™ website



An example of (+) agglutination from this experiment

Statistical Analyses

For each *Salmonella* enumeration date, the average of two 1 mL field duplicate samples was taken and multiplied by 100 to represent the number of suspect *Salmonella spp.* present in that respective stream per 100 mL standard volume. All enumerations of total coliforms and *E. coli* were also determined with respect to 100 mL volumes per convention.

A one-way analysis of variance (ANOVA) was used to determine differences between pooled counts of *E. coli* and *Salmonella spp*. (dependent variables) from all sample sites and dates. Bacterial counts from individual stream sites were then compared using student t-tests. F-tests were conducted to compare the standard deviations in counts of Salmonella spp. and E. coli for each site. The F-test results allowed determination of the correct student t-test equation to be used in comparing bacterial counts from the individual sites.

The least-squares method of regression analysis was conducted using Log_{10} transformed values to determine the degree of correlation between counts of *Salmonella spp.* and *E. coli* across the term of the study.

Results

Table 1 presents the raw data of paired bacterial counts obtained to date per 100 mL volumes. Figures 1 and 2 illustrate differences in bacterial counts per sample date at the two sampling sites for which we have the most data – Say5 (Fig. 1; n=8) and GRE16 (Fig. 2; n=8). The APP2 site contains only 5 sample dates and is not included. Table 2 presents the means for pooled data as well as for each site.

As shown in Table 3, one-way ANOVA results showed that the variances differ significantly (p<0.05) between the pooled bacterial counts.

Table 1-Pooled Raw Enumerations per 100mL

E. coli	*Salmonella spp.*
198	6800
192	500
148	3300
688	2100
408	3300
336	3500
128	4200
188	3000
344	800
256	600
200	11800
840	3700
416	3200
384	5100
504	5000
1040	2600
176	1400
100	3400
192	2100
88	2900
184	2100

Table 2. Measures of central tendency: pooled and site-by-site mean ± std dev

	E. coli	*Salmonella spp.*
Pooled data	333.8±251.7	3400±2464.8
APP2	148±49.8	2380±779.1
GRE16	498±293.2	4100±3534.7
SAY5	285.8±188.7	3337.5±1791.2

Table 3. One-way ANOVA results of pooled bacterial counts

Source	DF	SS	MS	F	P
Species	1	9865.88	9865.88	28.38	<0.0005
Error	38	13209.88	347.63		
Total	39	23075.76			

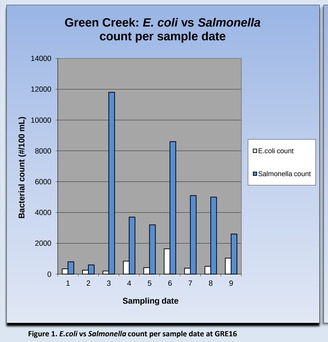


Figure 1. *E.coli* vs *Salmonella* count per sample date at GRE16

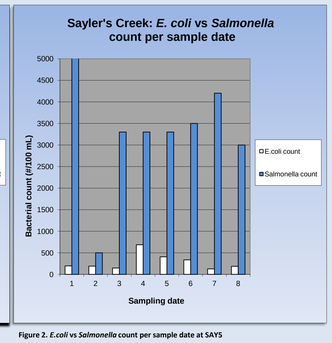


Figure 2. *E.coli* vs *Salmonella* count per sample date at SAY5

F-tests were completed on both the pooled raw data and on the individual sites alone except for APP2. All of the results from the F-tests showed that the standard deviations were all significantly different (see Table 4).

Table 4. F-test Results from Pooled Raw Data and Individual Sites

Data Set	$F_{Calculated}$	F_{Table} Critical Value	Level of Significance
Pooled Raw Data	95.86	2.12	p<0.05
Raw Data from SAY5	90.13	3.79	p<0.05
Raw Data from GRE16	145.39	3.79	p<0.05

Table 5 shows the results of the student t-tests that were completed on the individual sites alone except for APP2. All of the student t-test results showed that the means were all significantly different.

Table 5. Student t-test results from pooled raw data and individual sites

Data Set	$t_{Calculated}$	t_{Table} Critical Value	Level of Significance
Pooled Raw Data	5.8114	1.9600	p<0.005
Raw Data from SAY5	4.7920	2.2620	p<0.005
Raw Data from GRE16	2.8724	2.3650	p<0.025

Regression analysis was performed using the raw data set (see Figure 3) and Log_{10} transformed pooled data set (see Figure 4). An R^2 value of 0.0041 was obtained with a correlation coefficient of -0.0639 for the raw data analysis which is not significant (p>0.05). An R^2 value of 0.0905 was obtained with a correlation coefficient of 0.3008 for the Log_{10} transformed data which is significant (p<0.05), showing that a weak correlation does exist between pooled *E. coli* and *Salmonella spp.* counts.

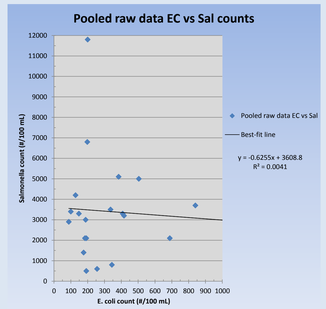


Figure 3. Pooled counts of *E. coli* and *Salmonella* raw data with best-fit line plotted

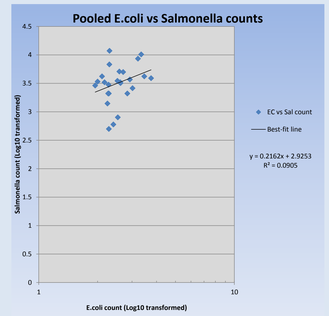


Figure 4. Pooled counts of *E. coli* and *Salmonella* using Log_{10} transformed data with best-fit line plotted

Discussion

Our results suggest that there is no correlation between raw numbers of the bacterial indicator *E. coli* and *Salmonella spp.* found within samples of our stream water (Table 1). Close examination of Table 1 reveals an order of magnitude difference between the indicator species and the potentially pathogenic species count from the same water sample. This difference may be explained by the reported difference in hardiness of the two genera of bacteria, with *Salmonella spp.* being quite hardy (e.g., resistance to environmental change) as compared with *E. coli.*
These results support other published data on the correlation between indicator bacteria and potentially pathogenic bacteria. Hörman et al. (2004) found no correlation between *E. coli* and select enteropathogens such as *Campylobacter spp.* Ahmed et al. (2008) and Schriewer et al. (2010) correlated fecal indicators, including *E. coli*, with potential pathogens, including *Salmonella spp,* concluding that there was poor correlation between the two groups. DePaola et al. (2010) examined the relationship between indicator bacteria and enteric viruses and found low correlation.

Using *E. coli* as indicator bacteria originated from a concern that sewage contamination of drinking water sources posed a significant health risk. This logic is sound in that sewage contamination contains high numbers of *E. coli* and if high numbers of *E. coli* are present, contamination is likely to have occurred. However, mounting evidence suggests that numbers of *E. coli* and other indicator bacteria show mixed results when correlated with pathogenic genera of bacteria, viruses, and protozoans.

Several suggestions have been offered to explain the lack of correlation between numbers of *E. coli* and pathogens. Luna et al. (2010) investigated the ability of *E. coli* to survive in marine sediments and concluded that *E. coli* are prevalent year-round. Solo-Gabriele et al. (2000) found that *E. coli* could effectively out-compete competitors in dry soil. Whitman et al. (2006) added that *E. coli* can persist in forest soils as well as in soil that is proximate to a major water source for long periods of time. These studies suggest that the ability of *E. coli* and other coliforms to exist as continuous non-point sources could potentially influence their effectiveness as indicator bacteria.

The purpose of indicator bacteria is to accurately gauge the quality of water by attempting to generally predict the degree of pathogenic presence. This purpose would benefit from future studies aimed at identifying better indicator bacteria that more closely correlate with specific pathogens. Relative to *Salmonella*, it may be advantageous to examine a hardier indicator bacterium that may correlate better with it. If, however, future data collections continue to support the correlation seen in Figure 4, it may be possible to predict *Salmonella spp.* numbers from *E. coli* numbers based upon a mathematical model effectively arguing for the continued use of *E. coli* as an indicator bacterium.

LONGWOOD UNIVERSITY
Department of Biological and Environmental Sciences