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Review Article

Evaluation of Total *Bacteroides sp.*, as an Alternative Indicator in Agricultural Water Quality

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ABSTRACT

Pre harvest irrigation water represents a key potential source of pathogenic bacteria during the production of fresh produce. Industry guidance metrics uniformly identify generic *Escherichia coli* (*E. coli*) as a practical and cost-effective indicator of fecal contamination in water. Although generic *E. coli* satisfies most characteristics of an ideal bacterial indicator, very limited correlation exists between the presence of *E. coli*, pathogen presence, and microbial risk to consumers following consumption. The Food Safety Modernization Act's Produce Safety Rule's Microbial Water Quality Profile is under abeyance due to concerns that there is not enough scientific evidence to support the rule in its' current form; thus, it is important to examine feasible alternative indicator organisms which could improve agricultural water quality assessment and the protection of public health.

This work examines the bacterial group, *Bacteroides* for prediction of pathogens in irrigation water samples (n=98) collected throughout Southern Arizona. Total *Bacteroides* sp. molecular markers were enumerated by quantitative Polymerase Chain Reaction, using: 1) a non- pathogenic *Bacteroides* sp. protocol (AllBac) published in the peer-reviewed literature and 2) a commercially available Pathogenic *Bacteroides sp*. enumeration kit (Primerdesign™ Ltd. Pathogen *Bacteroides*). Additionally, each water sample was culturally assayed for generic *E. coli* using EPA-approved Colilert® to assess correlations between *E. coli*, *Bacteroides sp*. markers and pathogens. Samples were additionally analyzed using Atlas® System Roka Biosystem to determine absence/presence of pathogenic *E. coli* O157:H7, STEC and *Salmonella*. Out of 98 samples, 31 tested positive for *E. coli*, but only 11 of the 31 samples exceeded the generic *E. coli* guideline of GM 126 MPN/100 mL for agricultural water. Results of the Allbac and Primerdesign™ Ltd. Pathogen *Bacteroides sp*. kit were then compared against the IDEXX Colilert-18© using linear regression to identify relationships between the two. The regression results were significant, p-value <0.02, suggesting that the assays for *Bacteroides sp*., performed as well as that for generic *E. coli*. However, the use of generic *E. coli* as an indicator (IDEXX), in this study, failed to 'indicate' pathogens in 80.6% of samples that were positive for pathogenic *E. coli* and/or *Salmonella* by ROKA, highlighting the well-established unsuitability of *E. coli* as an indicator.

Keywords: *E. coli*; Bacteroides; RT-qPCR; Geometric mean

INTRODUCTION

Since the implementation of the United States Environmental Protection Agency's (USEPA) Clean Water Act of 1972, regulations have focused primarily on the quality of recreational and potable water for the protection of public health [1]. Through this act, standard testing requirements for quantifying generic *Escherichia coli* (*E. coli*) using culture- based methods in waters was established. More recently, in 2007, the Arizona and California Leafy Greens Marketing Agreements (LGMA) were developed in response to produce outbreaks involving *E. coli* O157:H7 and leafy greens. Per LGMA requirements, similar methods and guidelines for irrigation water testing was adopted in an effort to mitigate the potential transfer of pathogens to leafy greens from agricultural irrigation water. While this effort was the first of its' kind, by outlining quantitative metrics for water quality used in leafy green production, it was only specific to crops grown in AZ and CA and left much to be desired from producers across the country. In 2011, the Food and Drug Administration's (FDA) Food Safety Modernization Act (FSMA) of 2011 (FDA, 2019) was implemented in an effort to standardize the growing, harvesting, packing and distribution of fresh produce across the country. While the FSMA relied heavily on USEPA water quality guidelines and methods, there is substantial concern that the water quality regulations set forth by the FDA are not adequate for identifying agricultural irrigation water contamination in an effort to mitigate potential human

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health risk from covered produce. This has been demonstrated by multiple foodborne outbreaks potentially linked to agricultural irrigation water; one recent outbreak of E.coli O157:H7 in romaine lettuce in 2018 originating from the Yuma growing region, which caused over 200 infections and 5 deaths across the United States as shown in Figure 1 [2,3].

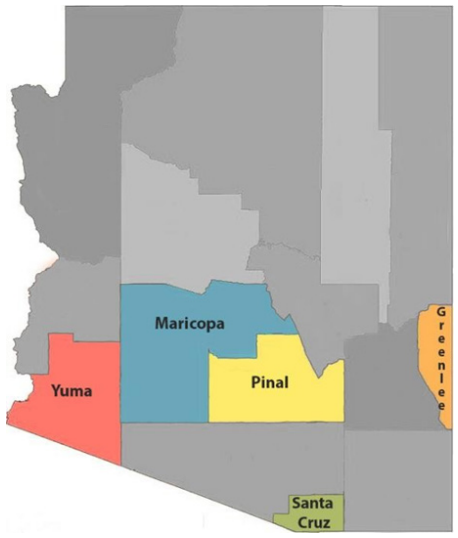

Figure 1: Map of the State of Arizona, USA. The counties where sampling occurred were Yuma, Maricopa, Pinal, Greenlee and Santa Cruz all located in Southern Arizona, USA.

Extensive research has identified poor quality agricultural irrigation water as a potential source of fresh produce contamination. The adaptation of the USEPA microbial water standards to agricultural water under FSMA identifies a maximum Geometric Mean (GM) and Maximum Statistical Threshold (STV) by analyzing 5 samples of groundwater over a 1 year period, and 20 samples over a period of 2 years not exceeding 4 years for surface water as shown in Figure 2 [4]. The GM is the central tendency of the water source with a maximum threshold of 126 CFU or MPN, of generic *E. coli* in 100 mL of water. The STV measures the variability of the water source roughly calculated at the 90th percentile with a maximum threshold of 410 CFU or MPN, of generic *E. coli* in 100 mL of water. Any irrigation water that exceeds this threshold must have corrective measures applied before crop application. *E. coli* was identified as the "ideal indicator organism" based on data from epidemiological studies on the probabilities of enteric illnesses functions in recreational waters. Yet, using *E. coli* as the Fecal Indicator Bacterium (FIB) for pathogens in water is not well supported by technical evidence [5-11].

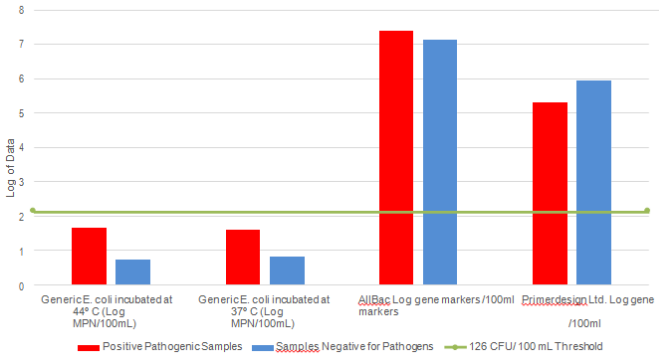

Figure 2: Comparing the geometric mean of confirmed pathogenic Salmonella and E. coli samples (n=31) to the negative samples (n=67) against log10 E. coli Colilert® MPN and *Bacteroides* sp. (AllBac) and Primerdesign™ Ltd. kit RT-qPCR data in log10.

One of the biggest limitations to the use of generic *E. coli* as an indicator of fecal contamination is its ability to replicate in the environment [12]. Studies done by Solomon et al. and Oliveira et al. concluded that *E. coli* O157:H7 had long term survival on leafy greens and possibly proliferated when applied at low concentrations in irrigation water. The studies, while having variable environmental conditions, were all able to demonstrate increases in *E. coli* O157:H7 on the leafy greens after irrigation application [13-15].

Multiple studies have also reported that generic *E. coli* dissatisfies another characteristic of an ideal FIB through a failure to correlate with the presence of pathogens reported in a study of irrigation water that, while 90% of the samples met the generic *E. coli* threshold of <126 CFU/100 mL, the same or increased levels of *Salmonella* were present in all samples. The study concluded that elevated levels of *Salmonella* could not be predicted by generic *E. coli*. This study and others support the need to consider alternatives to generic *E. coli* as the FIB mandated by current regulations [16-19].

As current efforts are underway to identify a more robust alternative indicator of fecal contamination and associated human health risk, *Bacteroides sp.* may present one option. *Bacteroides sp.* are obligate anaerobes and Gram-negative rods that are non-spore-forming. This bacterial group makes up to 40% of the organisms in fecal matter and 10% of fecal mass; such abundance indicates that *Bacteroides sp.* might represent a better FIB than generic *E. coli*. When comparing the six characteristics of an ideal indicator organism to both generic *E. coli* and *Bacteroides sp.* (Table 1), *Bacteroides sp.* is able to fulfill all the requirements. More importantly, it is unlikely that *Bacteroides*

Table 1: Characteristics of generic *E. coli* and *Bacteroides sp.*, as indicator organisms.

Characteristics of an ideal indicator organism	Common indicator: Generic *E. coli*	Indicator of interest: *Bacteroides sp.*
The organism is non-pathogenic and is naturally found in the intestinal micro flora of warm-blooded animals.	Both organisms are present in the intestinal micro flora and are considered non-pathogenic.	Both organisms are present in the intestinal microflora and are considered non-pathogenic.
The organism must be present when enteric pathogens are present and absent when pathogens are absent.	Is known to be present/ absent when pathogens are present/absent	Host Specific and will be present/ absent when enteric pathogens are present/absent
Should be able to live longer than pathogenic organisms	Known to survive in extreme conditions, and survive longer than pathogens	Known to survive longer than pathogens in water
Able to be easily and rapidly detected.	E coli can be detected easily and within ~24 hours using molecular and culturing methods	*Bacteroides sp.* can most easily be detected using molecular methods
Detected in larger quantities than the pathogen with a direct correlation to the pathogen.	Low detection rate when compared to *Bacteroides sp.*	Found in larger quantities when compared to *E. coli*
Should be resistant to proliferation in water.	Is known to proliferate in the environment	Unlikely to proliferate in water

sp., because of its requirement for anaerobic conditions, is able to multiply in the environment, unlike generic *E. coli*

The genus *Bacteroides sp*. has been long used in Microbial Source Tracking (MST) studies using Real-Time RT-qPCR (RT-qPCR) for differentiating sources of fecal contamination in water (Converse, et al.), However, the utility of *Bacteroides sp*. as a reliable FIB remains largely unexplored. In order for *Bacteroides sp*. to be deployed for use as a FIB in agricultural waters, routine agricultural water quality assessments and monitoring is needed. This study compared *Bacteroides sp*. to generic *E. coli* in assessing water quality using RT-qPCR, EPA-approved bacterial culturing methods, and comparison to the presence of a known pathogen (*Salmonella*). Ultimately, this work will contribute to improving the accuracy of predicting the presence of pathogens in irrigation water [20-22].

LITERARTURE REVIEW

Site location

Agricultural irrigation water samples were collected from five different counties in Southern Arizona: Pinal, Maricopa, Yuma, Santa Cruz, and Greenlee counties, between August and December 2015 (n=66) and January to May 2016 (n=32), a total of n=98 samples. Sample sites included a range in surface waters used for pre harvest crop management including ground to surface water reservoirs, irrigation district distribution main and lateral canals, and run off detention ponds also used seasonally for frost control and dust abatement Map of Arizona, USA.

Field analysis and sample collection

The surface water sources were collected according to standard protocols of the United States Geological Survey (USGS, 2006). Samples were collected in triplicate from each site in sterile 1 Liter Nalgene bottles (ThermoFisher Scientific Inc., Wilmington, DE, USA). During sampling, great care was taken not to disturb sediments. Physical parameters were also recorded using the Hach® Meter HQ 40d Multiprobe (Hach, Loveland, CO, USA) at the time of collection, including water temperature, conductivity, total dissolved oxygen, pH, and turbidity. Samples were transport on ice to the laboratory and stored at 4 degrees C until further processing. Sterilized deionized water was used as a field blank 'control' for all experiments.

Laboratory analyses

Samples were processed within 6 hours from the time of collection for using the EPA-approved Colilert® QuantiTray 2000 system (IDEXX Laboratories, Inc., Westbrook, ME, USA). This method estimates generic *E. coli* within a water sample using the most probable number method (MPN). Quanti Trays were run in duplicate for each collected water sample, with one tray incubated at 37°C and the other at 44°C for 18 to 24 h. While the standard incubation temperature for *E. coli* is 37°C, it has been suggested that 44°C may select for the growth of pathogenic *E. coli* and reduce background microbial flora. All samples, including controls, underwent the same analyses. All samples were incubated for 18 to 24 h according to manufacturer's instructions. Wells fluorescing blue under ultraviolet light were counted as presumptive for generic *E. coli*, providing estimates of MPN per 100 mL. During analysis 32 samples did not reach temperatures of either 37°C or 44°C due to equipment failure [23,24].

Molecular analyses

Water samples were refrigerated (4°C) until processing (3 days or less). In the laboratory, 250 mL of each water sample was filtered through a 0.45 µm filter (Millipore, Billerica, MA, USA). To calculate DNA recovery efficiency, the filter was then spiked with 100 µg/mL of Ultrapure™ Salmon Sperm DNA (LifeTechnologies Corporation, Carlsbad, CA, USA), followed by processing for DNA extraction using Power Water MoBio kit (MoBio Inc., Carlsbad, CA, USA). Nucleic acid concentration and purity was assessed from all extractions using the Nanodrop Lite™ spectrophotometer (ThermoFisher Scientific Inc., Wilmington, DE, USA). To identify the presence of *E. coli* O157:H7, 100 mL of each water sample was analyzed using the Atlas® System Roka Biosystem (Roka Bioscience, Inc., Warren, NJ, USA), utilized in the food safety industry because of its accuracy and efficiency of detecting pathogens using molecular methods. These data were utilized as confirmation of pathogen presence in the water samples [25].

Real-Time quantitative PCR assays

Each water sample was analyzed by RT-qPCR for generic *Bacteroides sp*. (henceforth known as "AllBac") and a commercial RT-qPCR Pathogenic *Bacteroides sp*.kit (henceforth known as "Primerdesign™ Ltd"). The advantage of using a commercially available kit was that the primers included with the kit have been assessed for broad inclusivity of desired targets and exclusivity of known non-target genera. The methods for RT-qPCR, including primer concentrations and target temperatures, followed those developed by Layton et al. and Primerdesign™ Ltd.

In addition to the *Bacteroides sp*. RT-qPCR assays, the Skeeta 22 RT-qPCR assay was used to calculate DNA recovery in samples due to the variable water sources used. Skeeta 22 detects the ITS region 2 of the Target Region of salmon sperm DNA. Gene targets, primer and probe sequences, and amplicon size for the RT-qPCR assays are summarized (Table 2).

Table 2: Quantitative PCR assays used in this study.

Assay	Primer/probe name and sequence [5'-3]	Locus	Size of Product (bp)
AllBac	AllBac296f, 5'-GAGAGGAAGGTCCCCCAC-3'	16S rRNA	106
	AllBac412r, 5'-CGCTACTTGGCTGGTTCAG-3'		
	AllBac375, 5'-[FAM] CCATTGACCAATATTCCTCACTGCTGCCT-[MGBNFQ]-3'		
Skeeta22	SkeetaF2: 5'-GGTTTCCGCAGCTGGG-3'	rRNA ITS region 2	77
	SkeetaR2: 5'-CCGAGCCGTCCTGGTC-3'		
	SkeetaP: 5'-[6-VIC]-AGTCGCAGGCGGCCACCGT-[TAMRA]-3'		

All samples were run in triplicate for AllBac and Primerdesign™ Ltd., while only a single reaction was run for the internal control of salmon sperm DNA. Each assay was performed as a singleplex and each reaction contained 10 µL 10 X PCR buffer (Sso Advanced Universal Probes Supermix, BioRad, Hercules, CA, USA), 400 nM of each forward and reverse primer, 200 nM probe, 5 µL extracted DNA, and DNase-free water to bring to 20 µL volume. The assays were performed using the Applied Bio systems Step One Plus™ Real-Time PCR System (Thermos Fisher Scientific Inc., Foster City, CA, USA). Amplification parameters for all assays had the same temperature profile as follows: denaturation at 95°C for 2 min, followed by 45 cycles of denaturation at 95 °C for 15 sec, annealing at 53 °C for 30 sec, extension at 72°C for 30 sec [26,27].

Standard curves for quantification were established using target *Bacteroides sp*. 16S rDNA fragments cloned into plasmid pUC19. Ten-fold serial dilutions of the plasmid DNA were utilized, and the resulting standard curves had limits ranging from 3 to 300,000 Genome Equivalent Copies (GEC) per reaction. Using the standard curves, cycle quantification (Cq) values from each extracted sample were converted into GEC per 100 mL sample processed.

Statistical analysis

Statistical analysis was performed using Mini Tab Statistical Software 19 (Minitab Inc., State College, PA, USA). Gene target values (Cq) for *Bacteroides sp*. were determined from the corresponding standard curve and MPN for *E. coli* and was log-transformed to allow for comparative statistical analyses. Logistic regressions were performed between *Bacteroides sp*. and generic *E. coli* from IDEXX Colilert®. For Colilert® at 37°C and 44°C against RT-qPCR results was done using a linear regression. The P-value of <0.02 was considered significant.

RESULTS AND DISCUSSION

Geometric mean ± standard deviation and linear regression results of Colilert® and water quality measurements. The summary of the geometric mean and standard deviation of Colilert® generic *E. coli* incubated at 37°C and 44°C by county are in Table 3. No difference was found in the detection of generic *E. coli* MPN when comparing Colilert® incubated at 37°C and 44°C (R2 of 68.2%; P<0.001).

Water quality measurements for turbidity, water temperature, pH, dissolved oxygen, and conductivity are provided (Table 4). No significant relationships were found between water quality measurements and the Colilert® generic *E. coli*, with the exception of water temperature and Colilert® generic *E. coli*, which showed a weak, but statistically significant relationship (R2 of 7.21%; P=0.014) (Table 5). This finding is supported by the Q10 model, which identifies survival rates of *E. coli* based on the temperature Blaustein, Pachepsky, Hill, Shelton, & Whelan. Lothrop et al. suggested that agriculture water collection should be done within a very closely regulated water temperature window to provide a more comprehensive overview of the microbial quality of the water [28].

RT-qPCR quality control metrics and DNA isolation efficiency

The RT-qPCR standard curves for all three assays, using standards from 3 to 300,000 GEC, showed a high degree of linearity, with an R2 of 99.7% ± 0.002 and an average slope of -3.40 ± 0.023 for the AllBac assay; R2=98.1% ± 0.010 and a slope of -3.34 ± 0.142 for Primerdesign™ Ltd. kit; and R2=98.0% ± 0.016 and a slope of -3.46 ± 0.155 for the Skeeta22 reference assay.

The reference assay Skeeta22, which identified the control of the salmon sperm DNA, yielded an average DNA extraction rate of 89.0% ± 6.0, based on the amount of salmon sperm DNA added to each sample and the calculation of DNA extracted from the filter, quantified using the standard curve.

Outcomes of the commercial manufactured pathogenic *Bacteroides sp*. kit using linear regression. Results obtained from all water samples using the commercial pathogenic *Bacteroides sp*. kit, Primerdesign™ Ltd. were compared to those from the well-established AllBac (Layton, et al.) assay using linear regression (Table 6). AllBac and Primerdesign™ Ltd. Cq results were statistically significant in every county and had a (R2 of 49.9%; P<0.001), suggesting that the two assays are comparable; however, AllBac revealed *Bacteroides sp*. which were, on average, nearly 2 logs higher than the pathogenic *Bacteroides sp*. detected by Primerdesign™ Ltd (Table 7). Since Allbac encompasses all *Bacteroides sp*. it can be presumed that the kit identifies Bacteroides fragilis, since 50% is found in fecal matter [28].

Table 3: Geometric mean ± the standard deviation of *E. coli* Colilert® (incubated at 37°C and 44°C)/ 100 ml by county and the linear regression analysis comparing *E. coli* Colilert® incubated at 37 °C and 44°C.

County	n	*E. coli* Colilert® (MPN/100 ml) incubated at 44 °C	n	*E. coli* Colilert® (MPN/100 ml) incubated at 37 °C	n	P-value	R^2
Yuma	36*	3.88 ± 10.65	45 †	4.63 ± 8.19			
Pinal	20	35.98 ± 181.68	16 †	40.05 ± 204.54			
Maricopa	12	8.52 ± 246.47	4 †	3.21 ± 2.18	66‡	< 0.001	68.20%
Santa Cruz	10	66.62 ± 49.37	10	72.16 ± 82.11			
Greenlee	5	25.60 ± 6.30	5	28.90 ± 2.71			

*: 14 samples were not processed due to equipment failure; †: 18 samples were not processed due to equipment failure; ‡: Only 66 samples were analyzed for regression due to equipment failure

Table 4: Geometric mean ± the standard deviation of water quality measurements by county.

County	n	Turbidity (NTU)	Water Temperature (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)
Yuma	51	3.23 ± 3.59	21.80 ± 7.05	8.23 ± 0.50	8.86 ± 1.79	1117.69 ± 227.88
Pinal	20	6.53 ± 9.84	21.41 ± 8.19	7.81 ± 0.87	8.19 ± 1.39	1118.52 ± 266.32
Maricopa	12	3.78 ± 29.76	23.42 ± 6.17	8.38 ± 0.56	8.89 ± 5.30	1089.08 ± 506.66
Santa Cruz	10	4.54 ± 15.37	22.03 ± 4.88	8.15 ± 0.66	8.06 ± 1.36	652.18 ± 101.86
Greenlee	5	94.40 ± 57.46	13.09 ± 2.98	8.31 ± 0.06	9.21 ± 1.41	471.71 ± 389.91

Table 5: Regression analysis of generic *E. coli* Colilert® MPN (incubated at 37°C and 44°C)/ 100 ml and water quality parameters.

IDEXX colilert®	n	Turbidity		Water temperature (°C)		pH		Dissolved oxygen (mg/L)		Conductivity (µS/cm)	
		R^2	P-value	R^2	P-value	R^2	P-value	R^2	P-value	R^2	P-value
Generic *E. coli* Colilert® (MPN/100 ml) incubated at 84* 44 °C		0.01%	0.945	7.21%	0.014	1.61%	0.25	3.64%	0	1.34%	0.294
Generic *E. coli* Colilert® (MPN/100 ml) incubated at 80 † 37°C		0.68%	0.468	5.62%	0.034	0.52%	0.523	3.31%	0	1.32%	0.311

*: 14 samples were not processed due to equipment failure; †: 18 samples were not processed due to equipment failure

Table 6: Regression analysis of the log10 of assays: AllBac and Primerdesign™ Ltd. molecular markers per 100 ml by county and total data.

County	n	AllBac and Primerdesign™ Ltd.	
		R^2	P-value
Yuma	51	24.90%	<0.001
Pinal	15*	48.20%	<0.001
Maricopa	8*	70.60%	<0.001
Santa Cruz	10	91.00%	<0.001
Greenlee	5	96.70%	<0.001
All Data	89	49.90%	<0.001

* 9 samples total did not amplify during qPCR

Table 7: Geometric mean ± the standard deviation of the log10 of assays: Allbac and Primerdesign™ Ltd. gene markers per 100 mL of sample by county and the qPCR efficiency (R2).

County	n	Log10 of AllBac markers/ 100 ml of sample	R^2	n	Log10 of Primerdesign™ Ltd. markers /100 ml of sample	R^2
Yuma	51	7.69 ± 0.69	0.980 ± 0.016	51	5.93 ± 0.56	0.999 ± 0.001
Pinal	20	5.52 ± 1.95		15 †	4.74 ± 1.56	
Maricopa	8*	7.06 ± 1.14		8 †	5.98 ± 0.86	
Santa Cruz	10	8.23 ± 0.66		10	6.13 ± 0.60	
Greenlee	5	9.01 ± 1.31		5	6.25 ± 1.09	

*: 4 samples total did not amplify during qPCR; †: 9 samples total did not amplify during qPCR

Linear regression of Colilert® and RT-qPCR

All the data from *E. coli* Colilert® and RT- qPCR were transformed to log10 per 100 mL of sample. This was done to compare results on the same scale, since RT-qPCR Cq can have a large range compared to the MPN of Colilert®. In table, generic *E. coli* Colilert® MPN/100 mL and both All Bac and Primerdesign™ *Bacteroides sp* (Table 8). RT-qPCR Cq were compared using linear regression. None of the data suggests that there is any correlation between generic *E. coli* Colilert® and *Bacteroides sp*. assays at 100 mL.

Positive pathogen samples compared to negative pathogen samples. The results from table show that, of the 98 samples tested for pathogens using the Roka system, 31 tested positive for pathogenic *E. coli* and/or *Salmonella* using Roka (Table 9). When compared to generic *E. coli* Colilert® results, only 8 of these samples exceeded the 126 MPN/100 mL threshold for incubation at 44°C and 6 samples for incubation at 37°C. All the samples that exceeded 126 MPN/100 mL were positive for pathogens using ROKA analysis. Though the incubation temperature that was reported to align with the presence of pathogens (44°C) had a slightly better correlation with samples positive for *Salmonella* and pathogenic *E. coli*, in the vast majority of cases, generic *E. coli* assayed using the Colilert® method did not exceed the 126 MPN/100 mL thresholds regardless of the presence of pathogens. The false negative rates of 74.8% for *E. coli* Colilert® incubated at 44°C and 80.6% for *E. coli* Colilert® incubated at 37°C demonstrates, as shown by other researchers, that reliance on *E. coli* as a sole FIB can result in erroneous data [13]. Comparing the RT-qPCR results of AllBac and Primerdesign™ Ltd to the confirmed pathogenic and non-pathogenic samples from ROKA analysis also indicated that there was no trend. In the AllBac assay RT-qPCR data set, confirmed ROKA pathogenic results were on average 2 log 10 higher than the ROKA non-pathogenic results. However, in the Primerdesign™ Ltd. assay RT-qPCR results, confirmed ROKA non-pathogenic results were on average 1 log 10 higher than the confirmed ROKA pathogenic results. While other studies have shown that generic

E. coli Colilert® to *E. coli* RT-qPCR assays provide comparable results, comparing generic *E. coli* Colilert® to *Bacteroides sp*. assays did not validate that *Bacteroides sp*. is a superior FIB (Tables 8 and 9).

An aspect that should have been considered in the design of this study was the incorporation of culturing *Bacteroides sp*. Comparison of a molecular method with culturing results can be problematic, as molecular work can easily identify genomic background DNA, thus overestimating the numbers of bacteria in water that are capable of causing disease. However, culturing *Bacteroides sp*. has been a challenge for laboratories, due to the fastidious growing conditions of this bacterial group [29-36].

Table 8: Regression analysis of the log10 of assays: Allbac and Primerdesign™ Ltd. gene markers/ 100 ml compared to log10 generic *E. coli* log10 MPN (incubated at 37 °C and 44 °C)/ 100 ml by county and total data.

Country	n	Allbac and generic *E. coli* Colilert® (incubated at 44°C)		n	Allbac and generic *E. coli* Colilert® (incubated at 37°C)		n	Primerdesign Ltd. and generic *E. coli* Colilert® (incubated at 44°C)		n	Primerdesign Ltd. and generic *E. coli* Colilert® (incubated at 37°C)	
		R^2	P-value		R^2	P-value		R^2	P-value		R^2	P-value
Yuma	37	2.80%	0.319	46	0.5%	0.629	37	0.0%	0.994	46	4.8%	0.146
Pinal	20	49.70%	0.001	15	6.2%	0.371	15	2.9%	0.544	10	4.4%	0.561
Maricopa	8	7.50%	0.511	4	70%	0.163	8	23.3%	0.225	4	56.8%	0.246
Santa Cruz	10	32.30%	0.087	10	35.2%	0.071	10	42.9%	0.04	10	47.6%	0.027
Greenlee	5	0.10%	0.957	5	18%	0.476	5	1.7%	0.833	5	23.3%	0.41
All Data	80	2.90%	0.13	80	0.0%	0.927	75	0.7%	0.468	75	2%	0.222

Table 9: Presence/absence results of pathogenic *E. coli* and/or *Salmonella* using the Roka compared to the samples that exceed 126 MPN/100 mL in generic *E. coli* Colilert®.

County	# of samples positive and negative for Pathogenic *E. coli* and/or *Salmonella*			# Of samples that exceed 126 MPN/ 100 ml generic *E. coli* Colilert® MPN			
	n	Positive	Negative	n	Generic *E. coli* Colilert® (MPN/100 ml) incubated at 44°C	n	Generic *E. coli* Colilert® (MPN/100 ml) incubated at 37°C
Yuma	51	8	43	37	0	46	0
Pinal	20	11	9	20	5	15	4
Maricopa	12	2	10	12	1	4	0
Santa Cruz	10	7	3	10	2	10	2
Greenlee	5	3	2	5	0	5	0
All Data	98	31	67	84*	8	80 †	6

*: 14 samples were not processed due to equipment failure; †: 18 samples were not processed due to equipment failure

CONCLUSION

This study has provided additional evidence that generic *E. coli* is not a suitable indicator of the presence of pathogens in water. While *Bacteroides sp*., has been suggested as an alternative indicator, results of this study indicate no difference between *Bacteroides sp*. and generic *E. coli* for the prediction of pathogens (*Salmonella* and *E. coli* O157:H7 and STEC) in agricultural irrigation water. If generic *E. coli* were a superior indicator, it would have outperformed *Bacteroides sp*., but there was little difference between the two.

In addition to the difference in quantitation of *Bacteroides sp*., another variance between the two molecular methods used in this work is the cost. Primerdesign™ Ltd. kits are more expensive, at $7.41 (USD) per reaction, compared to the average cost of a 96-well RT-qPCR assay of $0.89 (USD) per reaction (Hren ; Primerdesign Ltd.,). However, user skillset and equipment needs are similar for the two assays. A user with molecular skills will be needed to perform both the AllBac and Primerdesign™ Ltd. Kit, and both assays need the availability of a standard RT-qPCR machine. Overall, AllBac will provide ample results without incurring the cost of Primerdesign™ Ltd. *Bacteroides sp*. kits. However, if the research objectives require the quantification of pathogenic *Bacteroides sp*., of the two assays, only the Primerdesign™ Ltd. Kits will provide the necessary data.

While the issue of cost and scientific/technical skill level may be limiting factors, molecular analysis is becoming easier to understand, use, and afford. Molecular work is no longer solely confined to the laboratory; for example, the handheld MinION sequencer (Oxford Nanopore Technologies Ltd, Oxford Science Park, Cambridge, UK) can sequence DNA in a portable real- time device at a low-cost, with limited skills required, and can do this in field settings. With advancements in technology such as the Min ION, the meta genome of a water sample can be assessed, and as a result, reliance on FIB for assessment of water quality may no longer be necessary.

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