Typing *Vibrio parahaemolyticus* Without a Culture

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Abstract

Detection of foodborne pathogens can be greatly facilitated by nucleic acid assays but the usefulness of the methods for epidemiology and regulatory purposes is limited unless a culture is available. In 1998, the largest U.S. outbreak of *Vibrio parahaemolyticus* occurred involving oysters harvested from Galveston Bay, Texas, containing the O3:K6 serogroup, a new clone with an unusually high attack rate. During an environmental investigation of these oysters, colony lifts from two samples contained isolates that hybridized with a DNA probe for the thermostable direct hemolysin gene (*tdh*), a pathogenicity marker. No cultures from these colonies were isolated, thus, it was impossible to determine if the signals were actually from *V. parahaemolyticus*, or from the O3:K6 outbreak strain.

A PCR method was developed to amplify nylon or Whatman 541 filter-bound DNA previously hybridized with digoxigenin or alkaline phosphatase labeled probes. Signal positive colonies were cut from the filter and template was prepared by inserting a portion of the filter into the PCR reaction tube or by boiling filter cut outs in water. The PCR products from the filter-bound DNA were the same as those from broth cultures using primers for the *tdh* gene, the species-specific thermolabile direct hemolysin (*tlh*) gene, and the O3:K6 serogroup-specific ORF8. This method was applied to filter-bound DNA from isolates from the 1998 Texas outbreak samples. All colonies yielded appropriate PCR amplicons for *tlh* and *tdh* but there was no evidence of amplicons indicative of ORF8. Similar findings were obtained in a subsequent environmental survey of Alabama oysters using a modified procedure employing real-time PCR. The ability to confirm *tdh* signals by a second method and to subtype using filter-bound DNA in the absence of a viable culture can provide additional information vital to determining the public health significance of colony hybridization results.

Introduction

Vibrio parahaemolyticus is a naturally occurring gram-negative bacterium that is abundant in temperate and tropical estuaries and is a leading cause of gastroenteritis associated with seafood consumption. Illnesses are linked to consumption of raw seafood or cross-contamination after cooking or processing. After the discovery of *V. parahaemolyticus* in Japan during the 1950s, it was reported that nearly all clinical isolates, but few environmental or food isolates, produced a thermostable direct hemolysin (*TDH*) (3, 4). *TDH* is the product of the *tdh* gene; deletion of this gene results in a loss of virulence in animal models.

Until the late 1990s, sporadic cases or small outbreaks accounted for most *V. parahaemolyticus* infections. In 1995, a new clone of the *V. parahaemolyticus* O3:K6 serotype emerged in India and rapidly spread throughout southeast Asia. In 1998, it spread to the U.S. and caused the first reported *V. parahaemolyticus* pandemic (2). An open reading frame (ORF8) from a lysogenic filamentous phage appears to be universal in O3:K6 strains of *V. parahaemolyticus*.

In response to the oyster-associated outbreaks of *V. parahaemolyticus* O3:K6 in Texas during 1998 and in Washington state during 1997 and 1998, the Interstate Shellfish Sanitation Conference (ISSC) implemented an interim control plan for *V. parahaemolyticus*. One aspect of this plan called for monitoring levels of total and pathogenic V. parahaemolyticus in oysters at the time of harvest in states that had outbreaks. The method recommended by the ISSC involved spreading dilutions of oyster homogenate onto agar plates. After overnight incubation, colony lifts were prepared on Whatman 541 or nylon filters and hybridized with DNA probes labeled with alkaline phosphatase or digoxigenin, respectively. These probes targeted the thermolabile hemolysin gene (*tlh*) or the *tdh* gene to enumerate total or pathogenic *V. parahaemolyticus* respectively. This methodology was used in an environmental investigation in Galveston Bay, Texas, following the 1998 *V. parahaemolyticus* outbreak and in an oyster survey in Alabama. In each of these studies, colonies hybridizing with the *tdh* probe were observed on some filters but viable cultures were not always recovered. The lack of a culture precluded additional confirmation of *tdh*, or the ability to test for further characterization (i.e., serological test of O3:K6).

The objective of this study was to develop and evaluate PCR assays to confirm and characterize pathogenic *V. parahaemolyticus* using filter-bound DNA from colonies producing a hybridization signal with DNA probes for *tdh*.

Methods

Bacterial Isolates

Assay specificity and universality were determined for the *tdh* and ORF8 probes using the bacterial strains listed in Table 1 (facing page). These included *V. parahaemolyticus* strains previously tested for the *tdh* gene, strains representative of the new O3:K6 clone, and a variety of other *Vibrio* and non-*"Vibrio"* species. Bacterial strains were grown overnight at 35 °C in alkaline peptone water. A 1 mL aliquot was boiled for 15 min and centrifuged at 16,000xg for 1 min. A 1 mL aliquot was used as a template in the Real-Time PCR assay described below.

PCR Assays

The primers and probe used in Real-Time PCR to detect *tlh* and the *tdh*, genes of pathogenic *V. parahaemolyticus*, and the ORF8 region of the lysogenic phage found in the new clonal variant of O3:K6 strains are listed in Table 2 (page 4). Conventional PCR components and conditions for detection of the *tlh* and *tdh* genes were those described by Bej et al. (1999) (1). The components of Real-Time PCR were: 10 mM Tris-HCl, 9 mM $MgCl_2$, 50 mM KCl, 200 mM dNTP mix, 300 nM of each primer, 1.25 U Platinum™ Taq polymerase, and 50 nM of the fluorogenic probe. The PCR cycling conditions for all assays began at 94 °C for 2 minutes to denature the DNA and activate the hot start Taq polymerase. The *tlh* assay consisted of 50 cycles at 94 °C denaturation for 15 sec

Table 1 Pure Culture Species Tested With TaqMan™ Probes for Cross Reactivity

Species	Identification	*tdh*	*tdh*/ FAM	ORF8/ TET
A. hydrophilla	GCSL 110-3b	–	–	–
A. hydrophilla	1/97	–	–	–
B. subtilis	23234	–	–	–
B. subtilis	23857	–	–	–
E. coli	Famp	–	–	–
E. coli		–	–	–
L. mono	A1	–	–	–
L. mono	Scott A	–	–	–
L. mono	Vp-b	–	–	–
L. mono	2b	–	–	–
L. mono	3Vp	–	–	–
Salmonella	UAB	–	–	–
Salmonella spp.		–	–	–
V. alginolyticus	3093	–	–	–
V. alginolyticus	366	–	–	–
V. cholerae	ATCC 14103	–	–	–
V. cholerae	CDC F832	–	–	–
V. cholerae	FL-18	–	–	–
V. cholerae	UKB-70	–	–	–
V. cholerae	VRL 1984	–	–	–
V. cholerae	17-17	–	–	–
V. cholerae	20-21	–	–	–
V. cholerae	24-21	–	–	–
V. cholerae	25-16	–	–	–
V. cholerae	25-37	–	–	–
V. cholerae	25-62	–	–	–
V. cholerae	25-72	–	–	–
V. cholerae	40-14	–	–	–
V. cholerae	44-62	–	–	–
V. cholerae	72-24	–	–	–
V. cholerae	95-17	–	–	–
V. cholerae	133-29	–	–	–
V. cholerae	135-17	–	–	–
V. cholerae	140-16	–	–	–
V. cholerae	167-19	–	–	–
V. fluvialis	DAL 116	–	–	–
V. fluvialis	DAL 197	–	–	–
V. fluvialis	DAL 506	–	–	–
V. fluvialis	DAL 1678	–	–	–
V. fluvialis	DAL 1825	–	–	–
V. fluvialis	GCSL 358-2	–	–	–
V. fluvialis	1959-82	–	–	–
V. fluvialis	2386	–	–	–
V. fluvialis	2926	–	–	–
V. fluvialis	3282	–	–	–
V. fluvialis	4267	–	–	–
V. fluvialis	5125	–	–	–
V. fluvialis	5137	–	–	–
V. fluvialis	7214	–	–	–
V. fluvialis	11176	–	–	–
V. fluvialis	11961	–	–	–
V. hollisae	CFSAN 89A1960	–	–	–
V. hollisae	CFSAN 89A4206	–	–	–
V. hollisae	DAL 2039	–	–	–
V. hollisae	DAL 8391	–	–	–
V. hollisae	DAL 8393	–	–	–
V. hollisae	DAL 8395	–	–	–
V. hollisae	SPRC 8397	–	–	–
V. metschnikovi	ATCC 7708	–	–	–
V. metschnikovi	2908-8	–	–	–
V. metschnikovi	2360A	–	–	–
V. metschnikovi	2068	–	–	–
V. metschnikovi	2362	–	–	–
V. metschnikovi	2375	–	–	–
V. metschnikovi	2376	–	–	–
V. metschnikovi	2476	–	–	–
V. metschnikovi	2468	–	–	–
V. metschnikovi	2477	–	–	–
V. metschnikovi	2480	–	–	–
V. metschnikovi	2484	–	–	–
V. metschnikovi	9798	–	–	–
V. metschnikovi	10917	–	–	–
V. metschnikovi	11572	–	–	–
V. mimicus	ATCC 33053	–	–	–
V. mimicus	59	–	–	–
V. mimicus	85	–	–	–
V. mimicus	C-158	–	–	–
V. mimicus	196	–	–	–
V. mimicus	291	–	–	–
V. mimicus	667	–	–	–
V. mimicus	709-P	–	–	–
V. mimicus	1531	–	–	–
V. mimicus	2227	–	–	–
V. vulnificus	CDC 9062-96	–	–	–
V. vulnificus	CDC 9063-96	–	–	–
V. vulnificus	CDC 9064-96	–	–	–
V. vulnificus	CDC 9067-96	–	–	–
V. vulnificus	CDC 9341-95	–	–	–
V. vulnificus	CDC 9342-95	–	–	–
V. vulnificus	CDC 9343-95	–	–	–
V. vulnificus	CDC 9344-95	–	–	–
V. vulnificus	CDC 9345-95	–	–	–
V. vulnificus	CDC 9346-95	–	–	–
V. vulnificus	CDC 9347-95	–	–	–
V. vulnificus	SPRC 1275	–	–	–
V. vulnificus	SPRC 10271	–	–	–
V. vulnificus	SPRC 10273	–	–	–
V. vulnificus	SPRC 10277	–	–	–
V. vulnificus	A-9	–	–	–
V. vulnificus	J-7	–	–	–
V. vulnificus	MO-624	–	–	–
V. vulnificus	VBNO	–	–	–
V. vulnificus	304	–	–	–
V. parahaemolyticus	CT 6628	–	–	–
V. parahaemolyticus	DAL 1094	–	–	–
V. parahaemolyticus	TX 2046	–	–	–
*V. parahaemolyticus***	U-5474	+	+	–
V. parahaemolyticus	Cliff- MA	–	–	–
V. parahaemolyticus	Vp oys	–	–	–
V. parahaemolyticus	520	–	–	–
V. parahaemolyticus	1163	–	–	–
V. parahaemolyticus	2655	–	–	–
V. parahaemolyticus	4037	–	–	–
V. parahaemolyticus	116194	–	–	–
V. parahaemolyticus	CPA11 091399	–	–	–
V. parahaemolyticus	CPB12 091399	–	–	–
*V. parahaemolyticus**	NY 3064	+	+	+
*V. parahaemolyticus**	NY 4092	+	+	+
*V. parahaemolyticus**	NY 4095	+	+	+
*V. parahaemolyticus**	NY 003372	+	+	+
*V. parahaemolyticus**	NY 003374	+	+	+
*V. parahaemolyticus**	TX 2029	+	+	+
*V. parahaemolyticus**	TX 2030	+	+	+
*V. parahaemolyticus**	TX 2071	+	+	+
*V. parahaemolyticus**	TX 2072	+	+	+
*V. parahaemolyticus**	TX 2103	+	+	+
*V. parahaemolyticus**	295-3	+	+	–
V. parahaemolyticus	1029	+	+	–
V. parahaemolyticus	DIA6 031699	+	+	–
V. parahaemolyticus	DIB11 031699	+	+	–
V. parahaemolyticus	DIB9 031699	+	+	–
V. parahaemolyticus	D1D12 031699	+	+	–
V. parahaemolyticus	DID7 031699	+	+	–
V. parahaemolyticus	DIF8 031699	+	+	–
V. parahaemolyticus	DIE12 052499	+	+	–
V. parahaemolyticus	DIH8 060899	+	+	–
V. parahaemolyticus	DIA9 070799	+	+	–
V. parahaemolyticus	CPA7 081699	+	+	–
V. parahaemolyticus	DIA2 122799	+	+	–
V. parahaemolyticus	DIA11 011100	+	+	–
V. parahaemolyticus	DIA8 012500	+	+	–
V. parahaemolyticus	DIA-6-1 020800	+	+	–
V. parahaemolyticus	DIA-6-1 031400	+	+	–
V. parahaemolyticus	DIE3 031400	+	+	–
V. parahaemolyticus	DIB-1 052300	+	+	–
V. parahaemolyticus	DIB-5 052300	+	+	–
V. parahaemolyticus	DIB-1 060600	+	+	–
V. parahaemolyticus	CPB-5 060600	+	+	–
V. parahaemolyticus	DIB-1 062000	+	+	–
V. parahaemolyticus	CPA-6 072500	+	+	–
V. parahaemolyticus	BAC-98-03255	+	+	–
*V. parahaemolyticus**	BAC-98-3372	+	+	+
*V. parahaemolyticus**	BAC-98-3374	+	+	+
*V. parahaemolyticus**	BAC-98-4092	+	+	+
*V. parahaemolyticus**	KX-V225	+	+	+
*V. parahaemolyticus**	VP-86	+	+	+
*V. parahaemolyticus**	AN8373	+	+	+

* - O3:K6 strains; ** - 'Old' O3:K6 strain

Table 2 Primer and probe sequences used in Real-Time PCR to detect a) the species specific thermolabile haemolysin gene (*tlh*) using *tlh*/FAM probe, b) the pathogenic species specific thermostable haemolysin gene (*tdh*) using the *tdh*/FAM probe, c) the lysogenic phage sequence (ORF8) specific for the O3:K6 epidemic clonal isolate of *Vibrio parahaemolyticus* using the ORF8/TET probe.

Name	Sequence
VPTLH-243Fa	5´-AACTTCTGCGCCCGAAGAG-3´
VPTLH-305R	5´-CGGTTGATGTCCAAACAAGGA-3´
VPTLHTMP-263R	5´-FAM-ACGGTTTCGTGAACGCGAGCG-3´
VPTDHS-312Fb	5´-AAACATCTGCTTTTGAGCTTCCA-3´
VPTDHS-386R	5´-CTCGAACAACAAACAATATCTCATCAG-3´
VPTDHSP337F	5´-FAM-TGTCCCTTTTCCTGCCCCCGG-3´
VPORF8-1178Fc	5´-GGGACAATGCGTTAGCAAACA-3´
VPORF8-1259R	5´-CCCACTTTGAAGCGCTCTTT-3´
VPORF8TMP1200F	5´-TET-ACCTGCTTGGTTGTATTTTGTTCGCATTCAT-3´

with a combined annealing/extension step at 60 °C for 30 seconds. The *tdh* and ORF8 assays consited of 50 cycles at 94 °C denaturation for 10 sec and a combined annealing/extension step at 60 °C for 12 seconds. The fluorescent signals were measured during each cycle of PCR (Real-Time) using the Smart Cycler® system from Cepheid. Reaction tubes that generated a fluorescent signal of 30 fluorescent units above baseline within 50 cycles were positive.

Filter Preparation

PCR assays were conducted first with bacterial cultures grown overnight in alkaline peptone water (1.0% peptone, 1.0% NaCl and pH 8.5). These broth cultures were spotted onto T1N3 agar (1.0% tryptone, 3.0% NaCl, and 2.0% agar) and incubated overnight at 35 °C. Colony lifts were prepared on nylon or Whatman 541 filters and hybridizations were performed with digoxigenin or alkaline phosphatase-labeled *tdh* probes, respectively, as previously described (5).

PCR on Control Filter Bound DNA

Nylon and Whatman filters were prepared that containing a few colonies of a Tx2103 strain (*tdh* and O3:K6+). A Whatman filter was prepared containing a *tdh*+, O3:K6(-) environmental isolate (Figure 2). These filters were used to test for the presence of *tlh*, *tdh*, and ORF8. Concentric circles were drawn around the Whatman filters to mark off 1/2 centimeter intervals. The DNA samples from Whatman 541 and nylon filters were removed on an approximately one square millimeter portion of the filter using a sterile scalpel. Samples of an equal size were removed on the Whatman filters every 1/2 centimeter, and every one centimeter on the nylon filters. These negative areas away from the suspect colony were absent of any hybridization signal. The cut portions of the filters were placed in a 0.5 mL PCR tube. Template from the filter cut-out was prepared by boiling the Whatman 541 filter cut-outs in 20 mL sterile distilled water and using a 1mL aliquot in the PCR; the remaining template was stored at –20 °C . Template from nylon-bound DNA was prepared by removing a smaller portion of the filter cut-out and was used directly in the PCR.

PCR on Filter-Bound DNA from Environmental Oyster Samples

An environmental study following the 1998 *V. parahaemolyticus* outbreak involving Galveston Bay oysters used nylon filters to prepare colony lifts of plated oyster homogenates. Colonies hybridizing with a digoxigenin-labeled *tdh* probe were enumerated. Filter cut-outs of four colonies with visible hybridization signal were examined with PCR assays for *tdh* and ORF8 as described above. An environmental study of Alabama oysters used Whatman 541 filters to prepare colony lifts of plated oyster homogenates. Colonies hybridizing with an alkaline phosphatase-labeled *tdh* probe were enumerated as previously described. In cases where a viable culture was not recovered, filter cut-outs of colonies demonstrating visible signal of hybridization with the *tdh* probe were examined with PCR assays to confirm the presence of a *tdh* colony. Negative controls for environmental samples consisted of removing cut-outs from colonies on the same filter that were absent of any hybridization signal as well as regions of the filter that did not contain any visible colonies or hybridization signal.

Results

In this study, both the *tdh* probe labeled with FAM (*tdh*/FAM) and the ORF8 probe labeled with TET (ORF8/TET) specifically hybridized with pure culture bacterial DNA from strains that possessed the *tdh* gene and were of the new O3:K6 serogroup, respectively (Table 1, page). The *tlh* probe labeled with FAM (*tlh*/FAM) hybridized with the Tx2103 isolate and the Alabama environmental *tdh*+ control isolate.

Real-Time PCR is a sensitive and quantitative test (Figure 1).

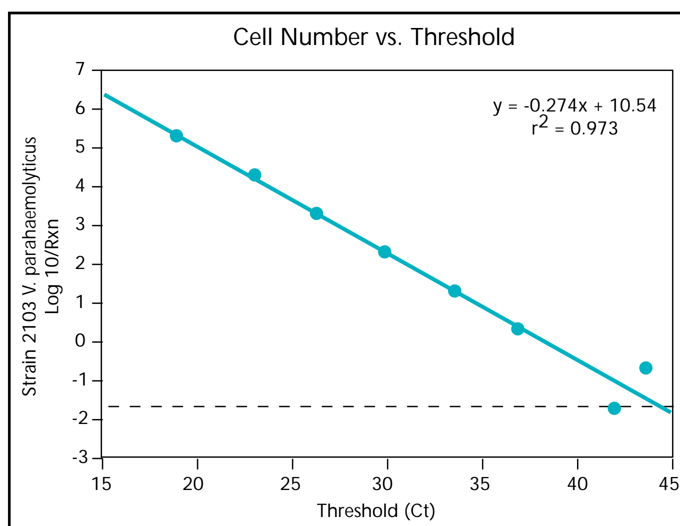

Figure 1: Graph showing the linear relationship between the Log value of a 10 fold dilution series of *Vibrio parahaemolyticus* and the Cycle threshold (Ct) value obtained using the *tdh*/FAM assay.

The optimized *tlh* and *tdh* assays could easily distinguish a positive colony from background contamination on the filters in as few as 4 cycles to 12 cycles.

Initial experiments showed that filter-bound DNA could be used as template in PCR. The quantitative ability of Real-Time PCR allows measurement and comparison of the differences in cycle threshold (Ct) values that result from low level DNA “bleed” from the higher levels of DNA that result with the actual colony (Figure 5 and Figure 6). Filter cutouts of four *tdh*+ colonies from the Galveston Bay, Texas filter (Figure 3) were examined by conventional PCR and generated amplicons indicative of *tdh*+ *V. parahaemolyticus*. No amplicons were detected with ORF8 PCR (results not shown).

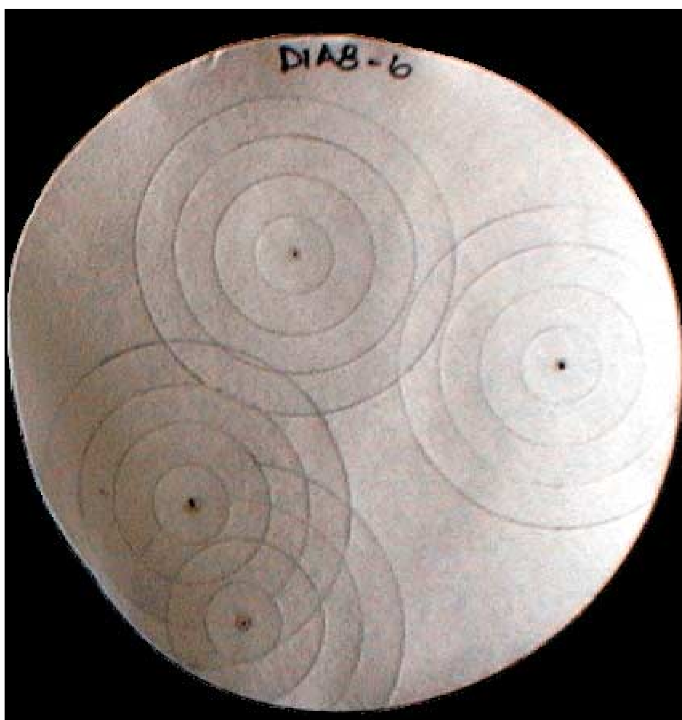

Figure 2: Whatman filter containing a *tdh*+ environmental isolate from Dauphin Island after hybridization with the tdh specific alkaline phosphatase probe. One millimeter square filter cut outs were removed at half a centimeter intervals and boiled in dH_2O. Samples taken included a cut out of the colony (A), and an equal sized cut out from sections B, C, and D. Results obtained using the Smart Cycler are shown in Figure 5. A similar experiment was run using the *tdh*+ O3:K6 strain Tx2103.

Boiled template of three suspected *tdh*+ colonies from Alabama oysters and two negative regions from each of three separate filters was examined by PCR. None of the three suspected colonies from the Alabama oysters gave a positive *tdh* or ORF8 signal above the background level of DNA contamination for each filter (Figure 4 and Figure 7 [page 7]).

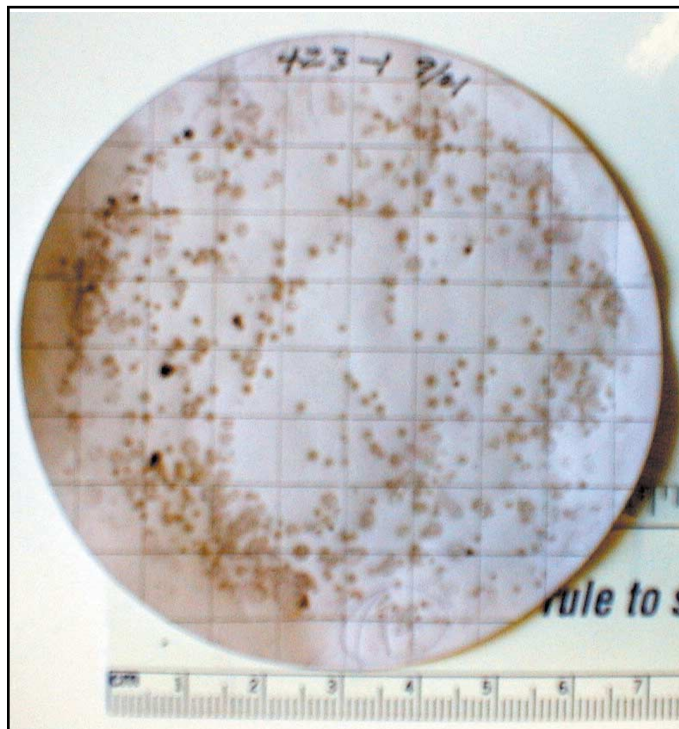

Figure 3: Nylon filter from the 1998 outbreak in Galveston Bay, Texas that was hybridized with a digoxygenin labeled *tdh* probe. Fourteen colonies were described as being *tdh*+. Initial testing of four of these colonies by conventional PCR revealed that the colonies were *tdh*+, and no O3:K6 strains were detected.

Figure 4: Whatman 541 filter from Dauphin Island, Alabama oyster survey hybridized with a alkaline phosphatase labeled *tdh* probe. Arrow indicates location of suspect *tdh* positive colony used for further typing by Real-Time PCR.

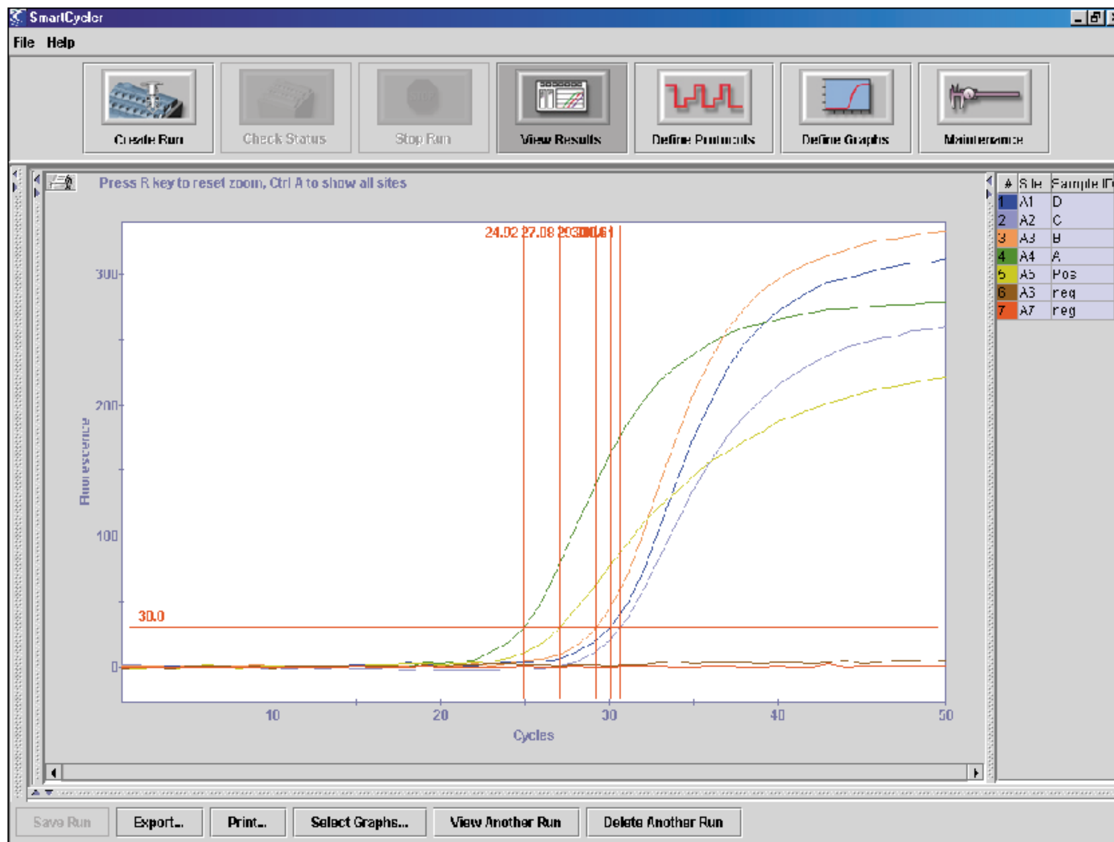

Figure 5: Smart Cycler result screen of diluted environmental Alabama isolate tested for "bleed" effect on Whatman 541 filters. Samples A–D were taken from the filter shown in Figure 2; Tx2103 boiled template was used as a positive control (Pos), and two samples with dH_2O as template were used as negative controls. Only sample A contained a positive hybridization signal on the Whatman 541 filter, with the remaining samples B, C, and D being absent of any hybridization signal or colony. All tested regions of the filter contained DNA that was recognized by the *tdh*/FAM probe.

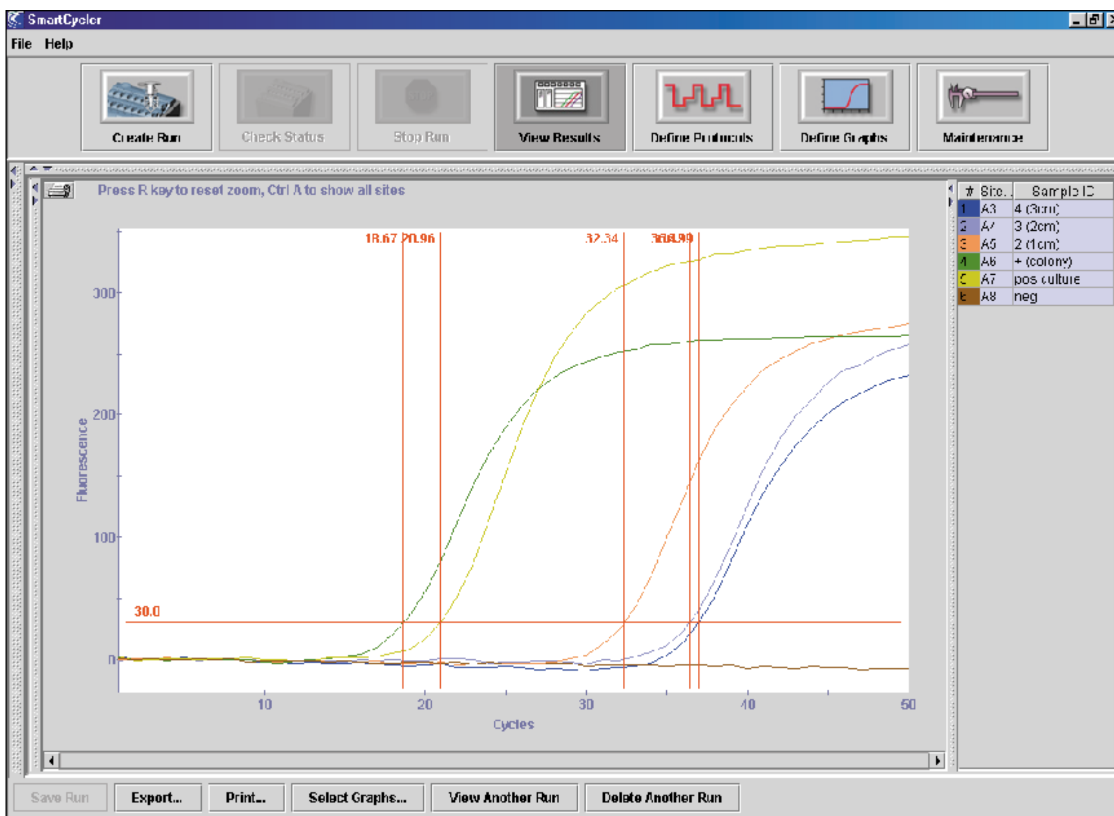

Figure 6: Smart Cycler result screen of diluted Tx2103 isolate to test for "bleed" effect on nylon filters. Cutouts of the nylon filter were taken in one centimeter increments away from the Tx2103 colony. Only the sample + contained a signal resulting from the hybridization of the DIG-labeled *tdh* probe, with the remaining samples 2, 3, and 4 being absent of any hybridization signal. All regions tested on the filter contained DNA that was recognized by the *tdh*/FAM Real-Time PCR probe.

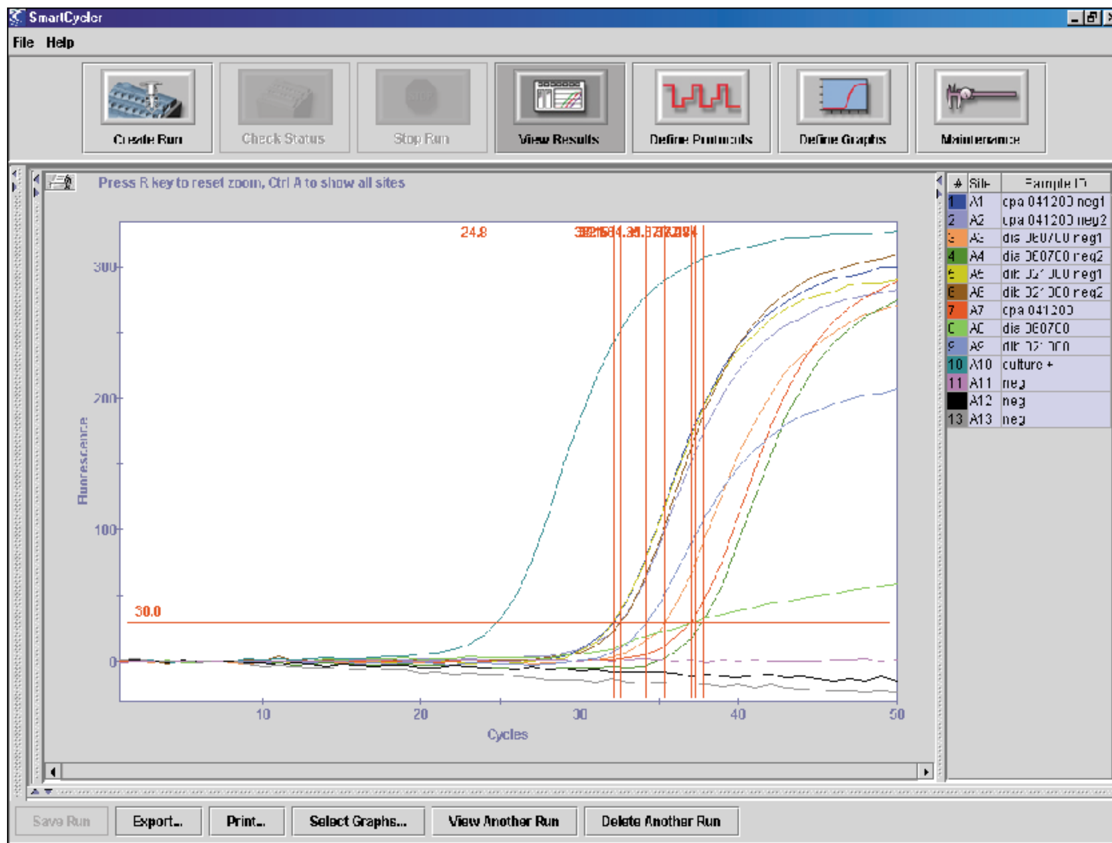

Figure 7: Smart Cycler result screen of three Whatman 541 Alabama oyster spread plates tested by Real-Time PCR using the *tdh*/FAM probe. Experimental samples from each filter consisted of two negative areas absent of hybridization signal (A1–A6) and one sample containing a suspected positive colony (A7–A9).

Discussion

Nucleic acid-based assays such as hybridization probes and PCR are sensitive, specific and rapid alternatives to traditional culture-based assays for identification and characterization of foodborne pathogens. However, the food safety community has been reluctant to rely on nucleic acid based assays for regulatory decisions because they do not yield viable cultures that can be subjected to further confirmation or characterization tests. This study demonstrated the utility of filter-bound DNA to serve as a template to confirm and further characterize bacterial colonies hybridizing to DNA probes that target specific virulence determinants.

The use of filter-bound DNA in a PCR allows for confirmation of suspect *tdh* and O3:K6 + colonies. This assists in removing the subjectivity encountered from visual examination of colony blots that is complicated by a weak signal and variations in colony size. In order for filter-bound DNA to be used as a secondary method of confirmation and classification, a few issues must be addressed. One of these issues is that DNA can “bleed” away from the colony during filter processing and contaminate other regions of the filter or other filters in the same batch.

The ability of Real-Time PCR to quantitate DNA based on Ct values allows measurement of the background level of DNA resulting from “bleeding.” This background level of contamination can then be compared to that from a signal generating colony that should contain a higher concentration of DNA targets.

A second issue is the variations in the sizes of the colonies being tested result in variations in the amount of DNA template used in the reaction mix. Spread plates from environmental samples often contain a large background of natural flora producing small colonies. If multiple assays are to be run on a small colony from a Whatman 541 filter, a colony can be cut out and boiled so that multiple assays are possible from the single colony. However, if the colony is on a nylon filter, the number of assays run is limited by the size of the colony tested. In our observations, nylon filters produced a more consistent and greater difference between positive samples and background.

Conclusion

- This methodology permits confirmation or classification of suspect colonies on filters when a culture was not recovered.
- The difference between (+) colonies and background was greater and more consistent with nylon than Whatman 541 filters.
- Multiplexing and further optimization of Real-Time PCR assays should provide confirmation of DNA probe results using the colony lift format.

// ACKNOWLEDGEMENTS

We would like to thank Drs. Rich Myer and Mike Bowen of the Bioterrorism Rapid Response and Advanced Technology Team at CDC for their assistance in design and development of primers and probes for use in Real-Time PCR.

Disclaimer

FDA as a government agency can not endorse any specific product, and that the poster represents preliminary work.

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