

Tentacle ProbesTM: Differentiation of Difficult Single-Nucleotide Polymorphisms and Deletions by Presence or Absence of a Signal in Real-Time PCR

BRENT C. SATTERFIELD,^{1,2} DAVID A. KULESH,³ DAVID A. NORWOOD,³
LEONARD P. WASIELOSKI, JR.,³ MICHAEL R. CAPLAN,^{1*} and JAY A.A. WEST²

Background: False-positive results are a common problem in real-time PCR identification of DNA sequences that differ from near neighbors by a single-nucleotide polymorphism (SNP) or deletion. Because of a lack of sufficient probe specificity, post-PCR analysis, such as a melting curve, is often required for mutation differentiation.

Methods: Tentacle ProbesTM, cooperative reagents with both a capture and a detection probe based on specific cell-targeting principles, were developed as a replacement for 2 chromosomal TaqMan-minor groove binder (MGB) assays previously developed for *Yersinia pestis* and *Bacillus anthracis* detection. We compared TaqMan-MGB probes to Tentacle Probes for SNP and deletion detection based on the presence or absence of a growth curve.

Results: With the TaqMan-MGB *Y. pestis* *yp48* assays, false-positive results for *Yersinia pseudotuberculosis* occurred at every concentration tested, and with the TaqMan-MGB *B. anthracis* *gyrA* assays, false-positive results occurred in 21 of 29 boil preps of environmental

samples of near neighbors. With Tentacle Probes no false-positive results occurred.

Conclusions: The high specificity exhibited by Tentacle Probes may eliminate melting curve analysis for SNP and deletion mutation detection, allowing the diagnostic use of previously difficult targets.

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Use of real-time PCR for infectious disease identification in a point-of-care clinical setting requires rapid analysis with a very low rate of false positives. Additionally, interpretation of the results must be straightforward, with little to no post-PCR analysis. Finally, because initial concentrations and identities of clinical samples are unknown, assay independence of initial concentration and purity is essential. Identical criteria are also important for use of DNA detectors in the field for detection of pathogens released for bioterror or biowarfare purposes.

The Roche LightCycler 2.0 and its field-adapted counterpart, the Ruggedized Advanced Pathogen Identification Device, are 2 devices with potential for point-of-care and field applications because of their automated software calling of growth curves. These instruments are based on a series of technologies designed largely by Wittwer et al. (1–3). The primary features of these devices include fast-cycling PCR with hot-air thermal cycling of reagents in glass capillaries. The detector software on both devices has the advantage of being intuitive to users without technical backgrounds in real-time PCR analysis because it can identify the presence or absence of a signal for automated target identification.

¹Harrington Department of Bioengineering, Arizona State University, Tempe, AZ.

²Arcxis Biotechnologies, Pleasanton, CA.

³United States Army Medical Research Institute of Infectious Diseases, Frederick, MD.

* Address correspondence to this author at: Arizona State University, PO Box 879709, Tempe, AZ 85287-9709. Fax 480-727-7624; e-mail Michael.Caplan@asu.edu.

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⁴Nonstandard abbreviations: SNP, single-nucleotide polymorphism; MGB, minor groove binder; RAPID, Ruggedized Advanced Pathogen Identification Device; USAMRIID, United States Army Medical Research Institute of Infectious Disease.

Automatic calling can be problematic, however, when genetic sequences are very similar [i.e., differ by a single-nucleotide polymorphisms (SNP)⁴ or deletion]. Assays designed to detect these genetic differences often generate low-level signal in the near-neighbor genotype, and this signal is pronounced when high concentrations of near-neighbor template are present. Although the difference in wild-type and low-level near-neighbor signals is discernable when concentration and sample integrity are known, signals from samples of dubious origin in which PCR efficiency may be inhibited could be more difficult to resolve, and growth curves from samples with unknown integrity and concentrations cannot be called with any degree of confidence. In these cases, signal generated from a high concentration of the near neighbor could be interpreted as a low level of wild-type organism. Many select agents have near neighbors whose genomes differ only by SNPs or deletions (4–6). Any signal from such a near neighbor would contribute to false positives with the detector software of the LightCycler or Ruggedized Advanced Pathogen Identification Device.

Recently our laboratories have designed and tested assays for chromosomal genes (*Bacillus anthracis gyrA* GenBank accession no. AY281534 and AY291535 and *Yersinia pestis yp48* GenBank accession nos. AL031866, NC_004088, NC_003143, NC_005810, and NC_006155) from 2 CDC Category A pathogens, *Y. pestis* (Bubonic plague) and *B. anthracis* (Anthrax) using Tentacle ProbesTM, which we recently showed increased specificity in SNP differentiation using a nonamplified fluorescence detection assay (7). In this study we apply Tentacle Probes to real-time PCR for the 1st time. These assays were selected because of ongoing difficulties with the selective detection of these genes in quantitative PCR using TaqMan minor groove binder (MGB) probes (8). *Y. pestis* differs from its near neighbor *Yersinia pseudotuberculosis* by a 25-base deletion in the *yp48* gene. Prior efforts to differentiate *Y. pestis* from *Y. pseudotuberculosis* with TaqMan-MGB probes demonstrated that post-PCR analysis was required (8). Although TaqMan-MGB probes have increased utility in differentiating genetic sequences differing by SNPs (9,10), their ability to successfully differentiate such sequences under more routine conditions is not clear. In the course of the studies by Chase et al. (8), 10 probes were synthesized to attempt the differentiation of *Y. pestis* from *Y. pseudotuberculosis* under real-time PCR conditions, without success.

B. anthracis differs from its near neighbor *Bacillus cereus* by an SNP in the *gyrA* gene (GenBank accession nos. AY291534 and AY291535). Hurtle et al. (11) synthesized 6 probes in an attempt to find a *B. anthracis* probe that demonstrated good specificity from its near neighbor *B. cereus* for this SNP. In the process, the annealing/fluorescence monitoring temperature was increased to 67 °C to improve specificity, a procedure that departed from the standard protocol and prevented multiplexing of the assay. Hurtle et al. (11) did attain specificity for *B.*

anthracis under laboratory conditions; however, in this study, we show that Hurtle et al.'s assay does not perform nearly as well under more challenging experimental conditions, such as boil preps, that are commonly used to screen environmental samples.

Tentacle Probes act through cooperative binding with both a detection and a capture probe designed around principles of specific cell targeting (12–16). These probes have been shown to have increased binding kinetics compared to standard stem-loop fluorescent DNA probes, to have concentration-independent melting curves, and to increase specificity without sacrificing sensitivity (7). These attributes allow the development of a binary style assay that generates a yes or no signal in the presence of wild-type or variant forms. In this study we compare the rate of false positives generated by TaqMan-MGB and Tentacle Probes in real-time PCR applications. We compared the previously developed and optimized TaqMan-MGB assay to single-iteration designs of Tentacle Probes for detecting the *gyrA* gene of *B. anthracis* and the *yp48* gene of *Y. pestis* (GenBank accession nos. AL031866, NC_004088, NC_003143, NC_005810, and NC_006155). These targets are chromosomal genes from CDC Category A pathogens that differ from their near neighbors by an SNP and deletion, respectively. This class of probes should be generally applicable to clinical genetic testing in which detection of SNPs and deletions is required.

EXPERIMENTAL PROCEDURES

Probe synthesis. Tentacle Probes are synthesized using standard controlled-pore glass chemistry (Biosearch Technologies) and contain both a capture and detection probe. The capture probe is a strand of linear DNA, and the detection probe contains a stem-loop structure with a fluorophore and a quencher. Each probe was dual HPLC purified and was not modified before use. The design of the Tentacle Probes was based on generalizations of the principles described by Satterfield et al. (7). Briefly, Mfold (<http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi>) was used to calculate melting temperatures of the detection probe stem, the capture probe-target duplex, and the detection probe-target duplex independent of each other. The targeted SNP/deletion was located in the center of the detection probe. We designed the detection and capture probes with predicted melting temperatures 5 °C below the assay temperature of 60 °C (55 °C) because the principles of cooperativity indicate that the individual binding sites should have affinities slightly weaker than the affinity required to achieve binding under the conditions present in the assay (7). The melting temperature for the stem-loop structure was chosen using the standard for Molecular Beacons, 7–10 °C above the assay conditions (70 °C) (17). TaqMan-MGB probes and primers are designed as reported in Chase et al. (8) and Hurtle et al. (11) using Applied Biosystems Primer Express software, version 2.0. Genomic DNA samples for *B. anthracis*, *B. cereus*, *Y. pestis*,

and *Y. pseudotuberculosis* were obtained from the United States Army Medical Research Institute of Infectious Disease (USAMRIID). PCR reagents were purchased from Idaho Technology. Probes and primers are summarized in Table 1.

yp48 assay. The real-time PCR conditions and probes developed by Chase et al. (8) for *yp48* differentiation were used and directly compared with Tentacle Probes targeting the same amplicon. The reaction conditions were 15 μ L of master mix (10.2 μ L deionized water, 2 μ L 10 \times reaction buffer in 50 mmol/L MgCl₂, 2 μ L of 10 \times dNTPs, 0.2 μ L each of 50 μ mol/L forward and reverse primers, 0.2 μ L of 10 μ mol/L probe, 0.2 μ L of *Taq* polymerase) with 5 μ L of template. TaqMan-MGB assay used *Taq* platinum polymerase with a 2-min denaturation at 95 °C, followed by 45 cycles of 95 °C for 0 seconds and 60 °C for 20 seconds. The Tentacle Probe assay used *Taq* TSP polymerase (exonuclease deficient) with a 2-min denaturation at 95 °C, followed by 45 cycles of 95 °C for 0 seconds, 60 °C for 10 seconds, and 70 °C for 10 seconds (mechanism in Fig. 1A). *Taq* TSP polymerase, which is exonuclease deficient, was used for Tentacle Probes to allow fluorescence monitoring at temperatures other than the annealing temperature, such as during the extension step. TaqMan-MGB probes required degradation, so *Taq* platinum polymerase was used in reactions with TaqMan-MGB probes. Because TaqMan-MGB probes were degraded, fluorescence monitoring at temperatures other than the annealing temperature was not beneficial. Standard dilutions of template were used from 20 copies to 20 000 copies with 3 replicates each for subsequent rounds

of PCR. Amplification products were run on a gel to verify successful PCR results.

gyrA assay. The real-time PCR probes developed by Hurtle et al. (11) for *gyrA* differentiation were used and directly compared with Tentacle Probes targeting the same amplicon under identical PCR conditions. Those conditions were 15 μ L of master mix (10.2 μ L deionized water, 2 μ L of 10 \times reaction buffer in 50 mmol/L MgCl₂, 2 μ L of 10 \times dNTPs, 0.2 μ L each of 50 μ mol/L forward and reverse primers, 0.2 μ L of 10 μ mol/L probe, and 0.2 μ L of Platinum *Taq* polymerase) with 5 μ L of template. The temperature cycles included a 2-min denaturation at 95 °C, followed by 45 cycles of 95 °C for 0 seconds and 60 °C for 20 seconds. Standard dilutions of template were used from 20 copies to 20 000 copies with 3 replicates each.

Both assays were assessed for specificity with boil preps of 29 environmental liquid collected air samples known to contain near neighbors to *B. anthracis*. Boil preps were prepared from 2 to 3 isolated colonies taken from overnight cultures on streaked plates. Colonies were removed from the plate, suspended in 500 μ L Dulbecco's PBS (8 g/L sodium chloride, 1.15 g/L sodium phosphate diacidic, 0.2 g/L potassium chloride, 0.2 g/L potassium phosphate monobasic), and centrifuged/washed twice. They were then suspended in 100 μ L deionized water at 95 °C for 15 min. The samples were centrifuged at 15 000g for 10 min, and the supernatant was collected for PCR analysis. The average nucleic acid concentration of each boil prep was 500 mg/L, and 5 μ L was used in each reaction. The reaction conditions for both Tentacle Probes

Table 1. Probe and target sequences with near neighbors are provided.^a

Target sequence and near neighbor

<i>B. anthracis gyrA</i>	5'-CCActctacgcatgaccatattcTATTCTTCACTAataaagggaaagtataccgTACG 1710
<i>B. cereus gyrA</i>	5'-CCActctacgcatgatcatattcTATTCTTCACTAataaagggaaagtataccgTACG 1710
<i>Y. pestis yp48</i>	5'-gagtattcgtctggggGCGTGCGGAAAtcgaggtcaggtg*****840gcacgTAAAGTGG
<i>Y. pseudoTB yp48</i>	5'-gagtattcgtctggggGCGTGCGGAAAtcgaggtcaggtgGATACCGCCGCCGCTCGTTCAGGTGagcacgTAAAGTGG865

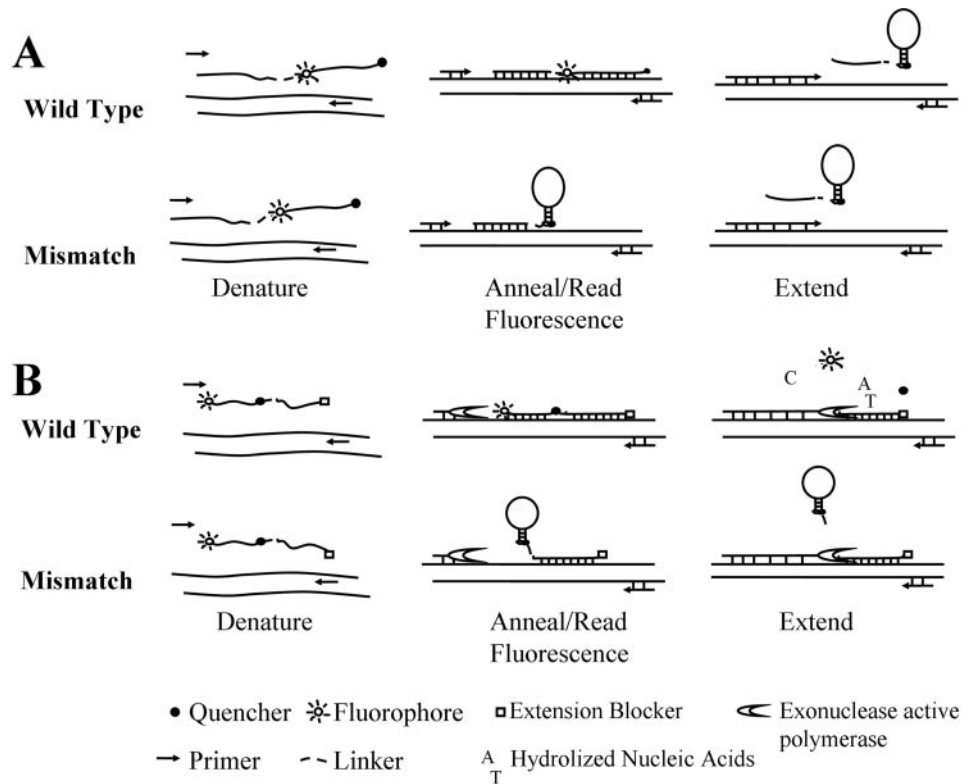
Primers and probes

<i>B. anthracis gyrA</i>	
Tentacle Probe	FAM-CTTCTA(CGCATGACCATATTC) gcgtagaag-BHQ-PEG9 -ATAAAGGGAAAGTATACCG-Carb3
TaqMan-MGB	FAM-CGCATGACCATATTC-MGBNFQ
Forward primer	GGGAACAATGATGATGATTCGT
Reverse primer	ACTCTGGGATTCATATCCTTCGT
<i>Y. pestis yp48</i>	
Tentacle Probe	GAGTATTCTGCTGGGG-PEG9-T(FAM)-ccc CG(AGGTTGAGGTGAGCACG) ctcgggga-BHQ
TaqMan-MGB	FAM-AGGTTGAGGTGAGCACG-MGBNFQ
Forward primer	GCAGGAAATGCGCAATGC
Reverse primer	GGGCGGATCCCCACTTTA

^a Carb and PEG represent the linker types of carbon chains or poly(ethylene glycol), respectively. The numbers following the PEG and Carb abbreviations represent the polymer length. FAM and BHQ are the fluorophore and quencher pair used. MGBNFQ is MGB and nonfluorescent quencher. Lowercase bases in Tentacle Probes represent bases added to help form the stem. Parenthesis around nucleotides in the Tentacle Probe is the region analogous to the corresponding TaqMan-MGB Probe. Lowercase bases in the templates represent probe binding regions. Lowercases in italics are regions where the capture probe binds, and nonitalicized text is where the detection probe binds. Bold bases represent polymorphisms and asterisks represent deleted bases.

Fig. 1. Mechanism of exonuclease-deficient (A) and exonuclease-active (B) PCR with Tentacle Probes.

Tentacle Probes consist of a linked capture and a detection probe. Under denaturing conditions, secondary structure of Tentacle Probes is removed and template is denatured. At the annealing temperature, the capture probe binds to the template providing affinity while the strong secondary structure of the detection probe provides specificity. The detection probe opens in the presence of the wild type, but remains closed in the presence of near neighbors. (A), because the probe is not digested, fluorescence can be read at any temperature or stage, not just at the primer annealing temperature, facilitating optimization of reaction specificity. The probe melts off at the extension temperature allowing primer extension. (B), the specificity of exonuclease-active PCR is limited to the primer annealing temperature because the probe hybridizes and is digested at this temperature, separating the fluorophore from the quencher, causing an increase in fluorescence. The detection region of a Tentacle Probe remains closed in the presence of a mismatch, causing the detection probe to be cleaved off, increasing the specificity of the assay. (In general, Tentacle Probes require binding to the proximal capture probe to open the detection probe; if the capture probe and detection probe are separated, then the detection probe cannot open.)



and TaqMan-MGB were 15 μ L of master mix (10.2 μ L deionized water, 2 μ L 10 \times reaction buffer in 50 mmol/L MgCl₂, 2 μ L of 10 \times dNTPs, 0.2 μ L each of 50 μ mol/L forward and reverse primers, 0.2 μ L of 10 μ mol/L probe, and 0.2 μ L of Taq Platinum polymerase) with 5 μ L of template undergoing a 2-min denaturation at 95 $^{\circ}$ C, followed by 45 cycles of 95 $^{\circ}$ C for 0 seconds and 60 $^{\circ}$ C for 20 seconds. A 2nd set of experiments was performed with an annealing temperature of 67 $^{\circ}$ C. One positive control containing 20 000 copies of the wild-type *B. anthracis* chromosome was run simultaneously with 29 boil preps from environmental samples known to contain near neighbors to *B. anthracis*. Amplification products were run on a gel to verify successful PCR results. Tentacle Probe reaction mechanism with exonuclease active polymerase is shown in Fig. 1B.

Results

The *Y. pestis* TaqMan-MGB exhibited results similar to those described previously (8). At all concentrations of near-neighbor *Y. pseudotuberculosis*, false positives occurred approximately 3 cycles later than detection of an equivalent concentration of *Y. pestis*. In contrast, Tentacle Probes yielded no false positives at any concentration tested (Fig. 2, A and B). Clean bands approximately 100 bases in size appeared for each PCR product when run in an agarose gel, indicating that lack of amplification was not the cause of the increased specificity (data not shown).

With both TaqMan-MGB and Tentacle Probes, the *gyrA* assay using 20–20 000 purified copies of both wild type

and variant produced no false negatives or false positives (Fig. 2, C and D). When boil preps of 29 environmental samples were used, however, TaqMan-MGB results included 21 false positives, whereas Tentacle Probes had no false positives for any of the samples (Fig. 3). A gel was run for all the PCR products from the boil preps for both TaqMan-MGB and Tentacle Probes (Fig. 4). The presence of amplification products in each indicated that the DNA was equally amplified in both experiments.

When Hurtle et al. (11) failed to achieve specificity of reaction with a 60 $^{\circ}$ C annealing temperature even after 6 designs of TaqMan-MGB, they used the best probe design at an increased annealing temperature, 67 $^{\circ}$ C. Accordingly, we repeated the TaqMan-MGB *gyrA* experiment with 29 boil preps of environmental samples at this high annealing temperature and still obtained 7 false positives for the 29 samples (data not shown) compared to zero false positive results for Tentacle Probes at the more conventional 60 $^{\circ}$ C.

Discussion

The ability to perform PCR without false-positive results is imperative for clinical and field diagnostics. Furthermore, the need to perform highly selective assays is complicated by the presence of near neighbors that often differ from the target organism by a single SNP or deletion. In this study we have shown improvements in 2 assays for the detection of priority infectious disease pathogens, with which false positives from near neighbors have been greatly reduced if not eliminated.

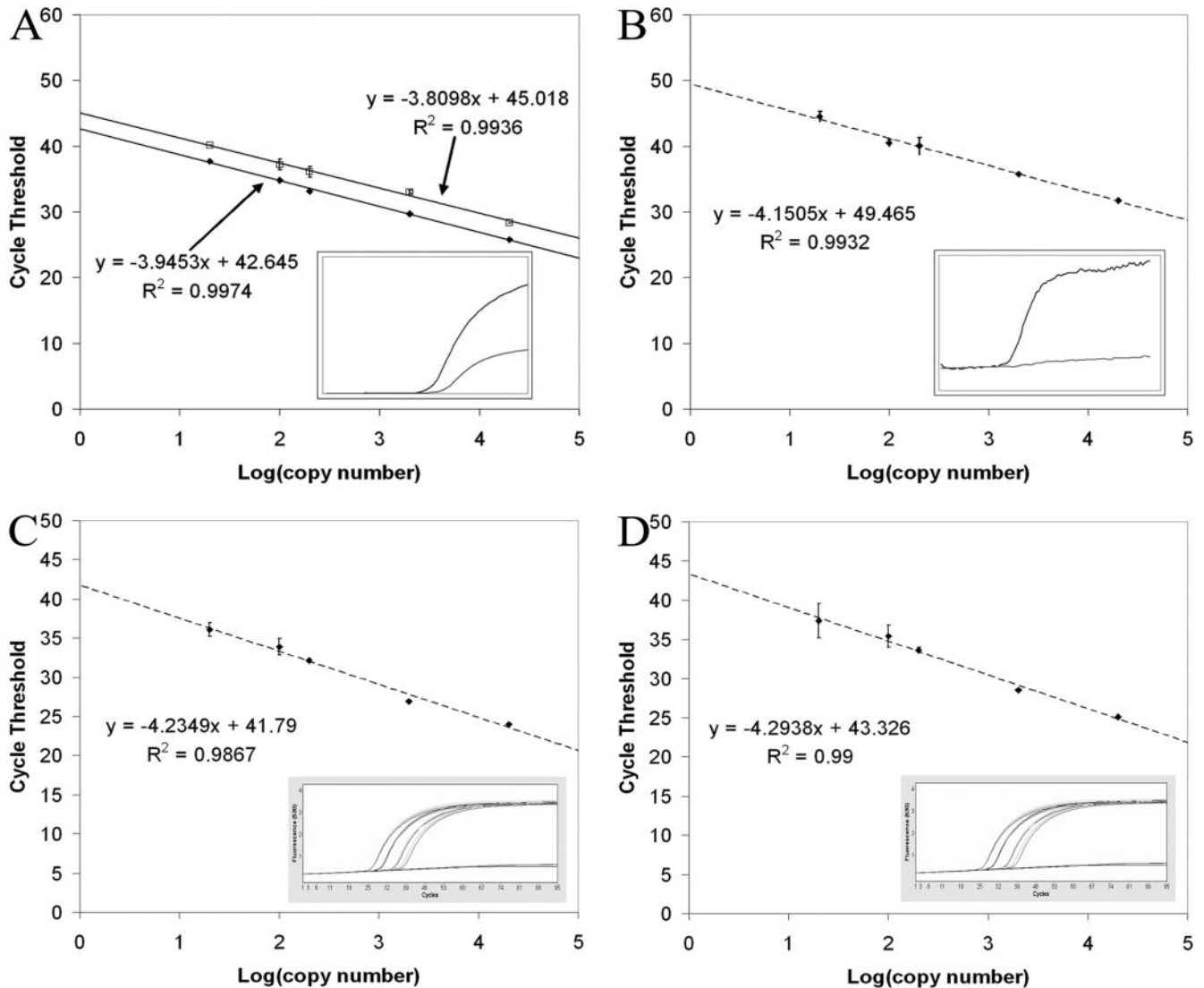


Fig. 2. Standard curve for *Y. pestis* (◆) and *Y. pseudotuberculosis* (□) for TaqMan-MGB assay (A) and Tentacle Probe assay (B).

Only 1 standard curve is shown for Tentacle Probes because no false positives were recorded at any concentration. PCR curves are shown for 20 000 copies of *Y. pestis* (top curve) and an equal number of *Y. pseudotuberculosis* (bottom curve) in the bottom right hand corner of each graph. Standard curve for *B. anthracis* for TaqMan-MGB (C) and Tentacle Probes (D) for purified samples. Representative PCR curves are shown for each in the bottom right hand corner of the graphs, where 4 concentrations of *B. anthracis* are shown in contrast to no signal for either probe type of 20 000 copies of *B. cereus*.

Y. pestis is an example of an organism for which few differences in the genome exist from its near neighbors (18). Although detection can be performed based on virulence plasmids (19–21), concerns regarding the development of genetically modified organisms and the implication that the plasmids alone are not responsible for the virulence of *Y. pestis* (5), require the addition of a chromosomal assay. The *yp48* gene contains one of the greatest differences between *Y. pestis* and *Y. pseudotuberculosis* a 25-bp deletion in *Y. pestis* (22).

It might be thought that detecting a 25-bp deletion would be easy; however, detecting the 25 deleted bases in the near neighbor does not rule out the presence of *Y. pestis*. The differentiation of the 2 species in this region is difficult for 2 reasons (Fig. 5). (a) The insertion is flexible,

allowing the 2 flanking sequences matching the probe to form a perfect match to the probe. (b) Additionally, 7 bases flanking the 5' end of the insertion are repeated in the 3' end of the insertion, causing exact matches to shorter probes, an especially problematic feature for probes that depend on short lengths for increased specificity. TaqMan-MGB is an example of a probe that requires short lengths for improved differentiation, thus explaining why false positives were present even after 10 design iterations of TaqMan-MGB probes (8). Because Tentacle Probes use a strong hairpin for enhanced differentiation and do not require short probes, they are ideal candidates for specifically detecting *Y. pestis*.

In spite of the progress Tentacle Probes achieve in differentiation without post-PCR analysis, there is still

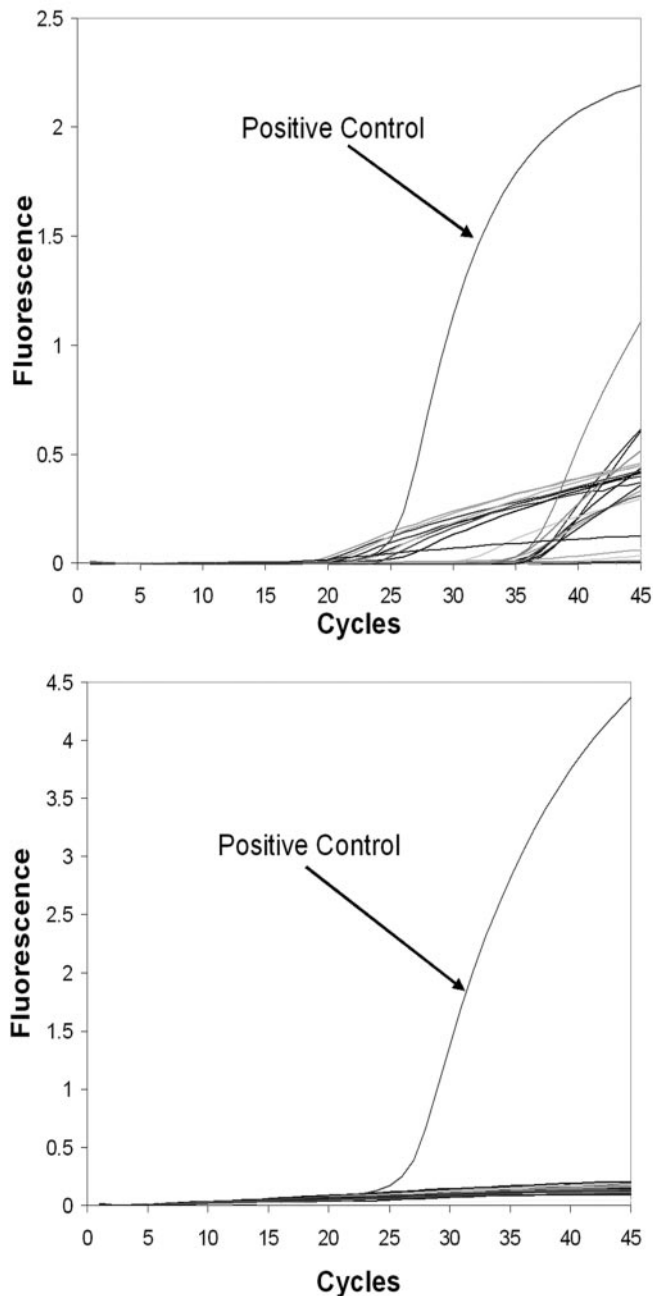


Fig. 3. Boil preps of 29 environmental samples of various strains of *B. cereus* and *B. thuringiensis* and 1 positive control (*B. anthracis*) were run for TaqMan-MGB (left) and Tentacle Probes (right).

TaqMan-MGB experienced 21 false positives out of 29 samples. Tentacle Probes had no false positives.

room for improvement. For example, the *Y. pestis* assay for Tentacle Probes required up to 7 additional cycles for detection (Fig. 2). Because this experiment was performed with exonuclease-deficient polymerase and growth curves were monitored during the extension step, we believe the longer cycle detection times were attributable to decreased amplification efficiency due to high probe melting temperatures. This explanation for loss in cycle

threshold was supported by repeats of the experiment with exonuclease-active polymerase, which resulted in similar cycle thresholds for both Tentacle Probes and TaqMan-MGB (data not shown). Further optimization of Tentacle Probes to avoid this limitation is possible, and it should be noted that the Tentacle Probes studied in this report were not optimized (1st-iteration design).

Anthrax detection can be performed by amplifying the virulence plasmids (23–25), but as with *Y. pestis*, there is concern over genetic modification of near neighbors and false negatives from *B. anthracis* without one or more of the plasmids. Moreover, *B. cereus* and *Bacillus thuringiensis* isolates have been found that harbor 1 or both *B. anthracis* plasmids with and without anthrax toxin genes (26–31). Accordingly, a chromosomal assay may augment the ability to distinguish *B. anthracis* from other environmental *Bacillus* isolates, but unfortunately there are few specific chromosomal targets to differentiate *B. anthracis* from other *Bacillus* species. Until now, the *gyrA* sequence in *B. anthracis* has appeared to be unique; however, the single-base mutation present in near neighbors has proved difficult to reliably reject even after 6 TaqMan-MGB probe designs (11). Although TaqMan-MGB has been used with greater success in the *gyrA* assay at an annealing temperature of 67 °C, this temperature departs from our standard protocol, precluding the option of multiplexing this chromosomal assay with virulence plasmid genes, and still reported 7 false positives out of 29 samples. For this reason, we chose to contrast the TaqMan-MGB assay and the Tentacle Probe assay at 60 °C.

TaqMan-MGB has been used with success to differentiate SNPs in other organisms (9, 32) and to detect *B. anthracis* via *gyrA* in laboratory samples; however, when boil preps of these samples were used, TaqMan-MGB reliability diminished sharply. One possibility for this failure is that variants other than the 49 strains of *B. cereus* and *B. thuringiensis* (testing reported in the original publication) were encountered (11). Another explanation is that the large amount of starting material present in boil preps may cause nonspecific amplification to occur, an explanation we consider unlikely, because gel images of the PCR product show that the samples that caused the most false positives (circled in Fig. 4) were predominately comprised of a single PCR product and were at approximately the same concentration as those that did not cause false positives.

Alternatively, TaqMan-MGB loss in specificity may be due to the presence of cell lysate, which contains salts, surfactants, and other molecules that may influence the binding behavior of the probe. Because the cells were lysed in deionized water, however, surfactant or chemical contamination is not possible, and salt carryover from previous steps should be minimal. Additionally, the DINAMelt server does not register any change in the predicted melting temperature for such a small amount of salt contamination (<http://frontend.bioinfo.rpi.edu/applications/hybrid/twostate.php>). A final hypothesis is

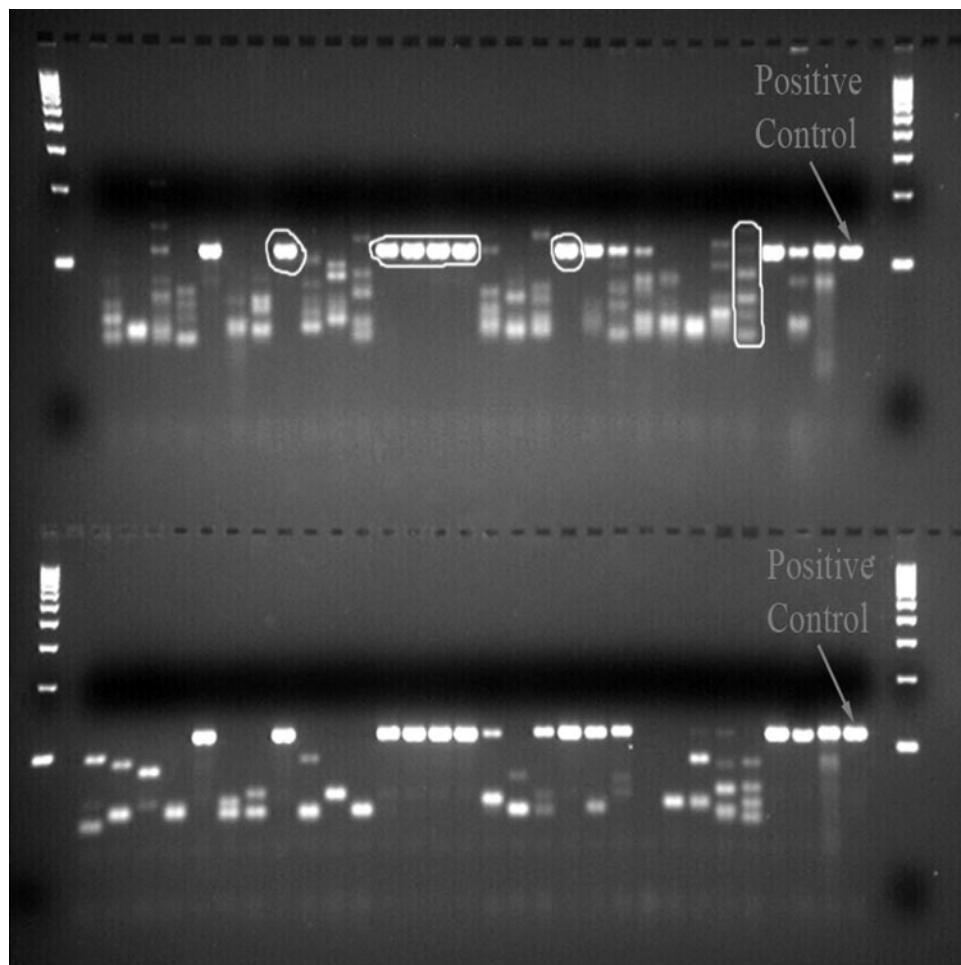


Fig. 4. DNA gel of PCR product from boil preps of the *gyrA* assay for both TaqMan-MGB (top) and Tentacle Probes (bottom) at 60 °C.

Samples that caused false positives at both 60 °C and 67 °C are circled. Ladder size is from 100 to 1500 bp in 100-bp increments. The presence of amplicon indicates that the lack of false positives in Tentacle Probes was not from failure to amplify but due to probe specificity.

the presence of cellular proteins and debris; however, the amount of these should be minimal after a rigorous centrifugation step. Any soluble proteins should have been denatured by heat during the lysis step. Furthermore, if an interaction with cellular proteins causes a PCR probe to fail, then its utility in clinical diagnostics is questionable.

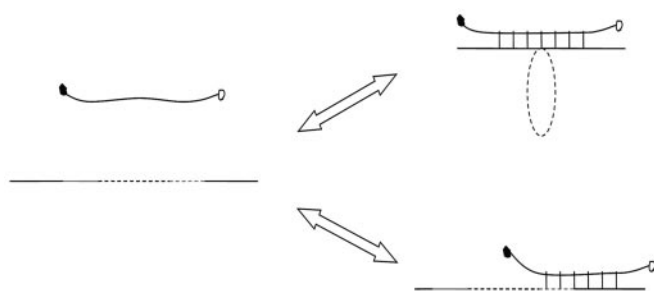


Fig. 5. Design of probes specific for deletions is difficult.

The 25 deleted bases in *Y. pestis* are present in *Y. pseudotuberculosis* (dashed line) and allow the 2 regions flanking the insertion matching the probe (solid lines) to come together, forming a perfect match, and consequently false positives, to a typical *Y. pestis* detection probe. Additionally, there is a repeat of the 5' flanking region (gray) in the insert, causing a perfect match to shorter probes and consequently false positives.

Whatever the cause of the failure mode of the TaqMan-MGB assay, Tentacle Probes maintained specificity under identical conditions. We previously reported Tentacle Probes with concentration-independent specificity, which prevents a loss of specificity, even in the presence of unexpected levels of nonspecific amplification (7). Second, the large stems in the hairpin of the detection probe provide a stabilized form that may be less susceptible to nonspecific binding in general. Although we cannot state the exact cause of the loss in specificity of TaqMan-MGB, we are certain that Tentacle Probes experienced no false positives under identical test conditions as are used with the TaqMan-MGB probes.

There are a variety of other probes and PCR techniques that have been successfully used in the differentiation of SNPs and deletions (33); however, Tentacle Probes is the only probe, to our knowledge, that exhibits cooperative binding between probe and target DNA. Simpler methods, such as allele-specific amplification, fail to confer the required specificity, because they do not completely inhibit amplification of the variant (34, 35). In clinical samples, for which starting concentrations and purity are unknown, delayed amplification cannot differentiate be-

tween the presence of a small amount of wild type and a large amount of variant. Of the hybridization probes, Scorpion primers and molecular beacons have shown excellent SNP differentiation in controlled laboratory environments (36–39). However, although TaqMan-MGB showed excellent SNP differentiation of the *gyrA* gene in a controlled environment across a panel of 49 strains of near neighbors without any false positives (11), it does not perform equally well with field samples, as demonstrated in this study. Similarly, although there are no data to indicate that Scorpion primers would yield false positives with field samples, there also are no data to indicate they will be successful.

The basic difference between Scorpion primers and Tentacle Probes is in the interaction of the stem-loop region with its target. Scorpion primers consist of a stem-loop detection region connected to a primer; the stem-loop region interacts with the extended primer resulting in a unimolecular detection event. Although similar in appearance, Tentacle Probes exhibit a cooperative interaction with the target DNA through their capture segment (compare to the primer region of the Scorpion primer) and also through their stem-loop region (instead of binding to the extended primer as in the Scorpion primer). Thus, Tentacle Probes have 2 interactions between the probe and the target DNA compared to the 1 interaction for Scorpion primers. As described above, we cannot be certain as to the cause of the improved performance of Tentacle Probes over TaqMan-MGB, but a strong candidate is the cooperativity generated by this 2nd interaction between probe and target DNA. If this hypothesis is correct, Tentacle Probes would likely exhibit advantages over Scorpion primers similar to the advantages over TaqMan-MGB.

In summary, we developed a 1st iteration Tentacle Probe design that detects 2 priority select agents without any false positives, in contrast to multiple iteration designs of TaqMan-MGB, which continue to generate false-positive results from near neighbors. The high degree of specificity attainable with Tentacle Probes will allow more precise identification of genetic mutations, including SNPs and deletions, without requiring melting-curve analysis.

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