Single Laboratory Validation of the BAX qPCR *Vibrio* Assay for Identification of Vibrio Isolates

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SUMMARY

Introduction: *Vibrio cholerae* (Vc), *V. parahaemolyticus* (Vp), and *V. vulnificus* (Vv) are well-documented human pathogens associated with seafood consumption. The FDA BAM recommends enrichment in alkaline peptone water (APW) followed by colony isolation on selective/differential agar. Biochemical testing or conventional PCR procedures are recommended for identification of suspect vibrio isolates**.** This study compares the BAX *Vibrio* assay to the BAM procedure for identification of vibrio isolates.

Methods: *Vibrio* cultures were grown in APW overnight at 35°C, streaked onto selective agars and confirmed biochemically as Vc, Vv, or Vp using API20E, VITEK and conventional biochemical tests. For the BAM PCR confirmation, a crude lysate was prepared by boiling the overnight APW enrichment and 2 µl was used as template for detection of *V. cholerae* cholera toxin (777 bp fragment of *ctx*AB), *V. vulnificus* spp.(519 bp fragment of *vvh*) and *V. parahemolyticus* (triplex assay for 450 bp fragment of *tlh* species specific marker, 500 bp fragment of *trh* virulence marker and a 270 bp fragment *tdh* virulence marker PCR products were visualized by gel electrophoresis as described in the BAM. For the BAX *Vibrio* assay, the manufacturer's instructions were followed for multiplex qPCR detection of Vc, Vv, and Vp.

Results: The BAX® *Vibrio* assay correctly identified 51/52 Vc isolates, 53/53 Vv isolates and 50/50 Vp isolates. Forty five near neighbor and non vibrios were negative for all three targets in the multiplex assay. Confirmation of isolates by biochemical testing and conventional BAM PCR showed equivalent results for Vv and Vp. The Vc PCR in the BAM identified that 16 of the isolates possessed the Vc toxin gene. Vc, Vp and Vv isolates that had one or more atypical reactions on the API20E or VITEK 2 Compact identification systems were either poorly identified or misidentified.

Significance: The results presented here demonstrate the BAX *Vibrio* assay is a reliable and rapid alternative to the BAM methods for identification of Vc, Vv, and Vp isolates.

INTRODUCTION

The genus *Vibrio* includes Gram-negative, rod or curved rod-shaped facultative anaerobes. Vibrios are naturally occurring environmental bacteria, present in almost all coastal waters of temperate and tropical regions of the world. (Kaysner, 2000). Many *Vibrio* spp. are pathogenic to humans and have been implicated in food-borne disease.

Vibrio spp. cause a significant number of foodbornre infections, usually from the consumption of raw or undercooked shellfish (DePaola and Kayser, 2004). *Vibrio* spp. can be carried by numerous sea-living animals, such as crabs or prawns, and has been known to cause fatal infections in humans during exposure. Pathogenic *Vibrio* include *Vibrio cholerae* (Vc), *V. parahaemolyticus* (Vp), and *V. vulnificus* (Vv) which are responsible for at least 75% of to seafood-related bacterial infections (Feldhusen, 2000).

The identification of vibrios based on phenotypic traits by classical microbiological methods has always been problematic (Oliver and Kasper, 1997). Molecular techniques have become a powerful adjunct to these classical methods. PCR, DNA hybridization techniques and real time PCR techniques have been developed for detection of pathogenic vibrios.

In the current study we evaluated the BAX multiplex Vibrio assay for identification of vibrio isolates as Vc, Vp or Vv.

Figure 1. *Vibrio parahaemolyticus* (left), *Vibrio cholerae* (center), and *Vibrio vulnificus* (right) on TCBS agar

MATERIALS AND METHODS

Culture preparation

Fifty two different strains of *V. cholerae*, fifty strains of *V. parahaemolyticus*, fifty three strains of *V. vulnificus* and 45 near neighbor and non vibrio bacteria were used in the evaluation. The near neighbor and non vibrio strains examined were: *Vibrio alginolyticus* GCSL-1, *Vibrio alginolyticus* GCSL-2, *Vibrio alginolyticus* GCSL-3, *Vibrio fluvialis* ATCC 11961, *Vibrio fluvialis* DAL 1678, *Vibrio fluvialis* GCSL-1, *Vibrio fluvialis* GCSL-2, *Vibrio fluvialis* GCSL-3, *Vibrio fluvialis* GCSL-4, *Vibrio fluvialis* GCSL-7, *Vibrio hollisae* GCSL 98A1960, *Vibrio metshnikovii* ATCC 10917, *Vibrio metshnikovii* ATCC 2477, *Vibrio mimicus* GCSL-1, *Citrobacter freundii* ATCC 8090, *Edwardsiella tarda* ATCC 15947, *Enterobacter aerogenes* ATCC 13048, *Enterobacter cloacae* ATCC 23355, , *E. coli* O157 43888, *E. coli* O157 ATCC 43895, *E. coli* O157 ATCC 35150, *E. coli* O45 SEA 13F73, *E. coli* O103 SEA 13D30, *E. coli* O26 SEA 13H58, *E. coli* O111 SEA 13D58, *Hafnia alvei* ATCC 13337, *Klebsiella pneumoniae* ATCC 13883, *Photobacterium damselae* GCSL-PH3, *Photobacterium damselae* GCSL-PH4, *Photobacterium damselae* GCSL-PH5, *Proteus hauseri* ATCC 13315, *Providencia rettgeri* ATCC 14505, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* California ATCC 23201, *Salmonella* Cholerauis ATCC 10708, *Salmonella* Gaminara ATCC 8324, *Salmonella* Montevideo ATCC 8387, *Serratia marcescens* ATCC 8100, *Serratia marcescens* GCSL-NV16, *Serratia marcescens* GCSL-NV17, *Shewanella algae* GCSL-NV15, *Shigella boydii* ATCC 9207, *Shigella dysenteriae* ATCC 13313, *Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 25931.

The bacteria were maintained at -70° C in peptone storage media supplemented with 50% glycerol. The *V. cholerae* and non vibrio strains were then transferred to Tryptone + 1% salt agar (T_1N_1) plates. The *V. parahaemolyticus* and *V. vulnificus* strains were transferred to Tryptone + 3% salt agar (T_1N_3) plates. All cultures were incubated at 35° C for 18 -24 hours.

Detection

After incubation, one well isolated colony (for all strains) was transferred to Alkaline Peptone Water (APW) and incubated overnight at 35° C for 18 -24 hours. The APW cultures were screened on the BAX® Vibrio system according to the manufacturer's instructions.

PCR was also performed from the APW cultures as described in the BAM for *V. cholerae* cholera toxin (777 bp fragment of *ctx*AB), *V. vulnificus* (519 bp fragment of *vvh*) and *V. parahaemolyticus* (triplex assay for 450 bp fragment of *tlh* species specific marker, 500 bp fragment of *trh* virulence marker and a 270 bp fragment *tdh* virulence marker. Product from each PCR was visualized by gel electrophoresis as described in the BAM and by microfluidic separation on the Agilent 2100 using Agilent DNA 1000 Lab Chips (Life Technologies, Foster City, CA).

Cultural Confirmation

All APW cultures were streaked to Thiosulfate-Citrate-Bile Salts-Sucrose agar (TCBS) and screened according to BAM chapter 9. The TCBS plates were then incubated at 35° C for 18 -24 hours. Cultures that failed to grow on TCBS agar were grown on TSA for additional testing.

After incubation, a well isolated colony from TCBS was transferred to T_1N_1 agar , TSA + 2% salt, T_1N_0 broth, and TSB + 2% salt. All media were incubated at 35° C for 18 -24 hours. Isolates from T_1N_1 agar were confirmed by PCR as outlined in the BAM and by the BAX® Vibrio multiplex assay. In addition to PCR, all isolates were identified using API 20E test strips and reagents (BioMerieux, Durham, NC) and VITEK 2 Compact with GN cards (BioMerieux). For each identification system, isolates were scored as a particular organism if the likelihood was determined to 51 % probability or greater. Additional tests performed were: string test, oxidase, and Gram reaction.

Statistical Analysis

McNemar's Chi Square (χ^2) analysis was performed to compare the results. A χ^2 value of 3.84 indicated a significant difference at p = 0.05.

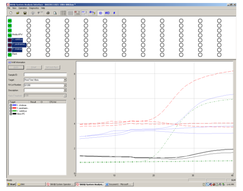

Figure 2. BAX® Vibrio multiplex for *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus*

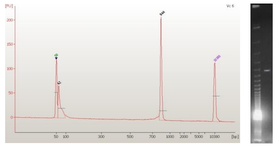

Figure 3. BAM PCR for detection of *Vibrio cholerae* cholera toxin (777 bp fragment of *ctx*AB), Agilent 2100 (left) and gel electrophoresis (right)

RESULTS

Table 1. Identification of vibrio isolates by BAX® Vibrio PCR Assay, conventional PCR BAM as outlined in the BAM (Chapter 9) on agarose or Agilent DNA chips, API20E and VITEK 2 Compact identification systems

Microorganisms	BAX	BAM PCR		API	VITEK
		Agarose	Agilent		
V. cholerae	51/52	16/52 (*ctx*AB)	16/52 (*ctx*AB)	48/52	51/52
V. parahaemolyticus	50/50	49/50	50/50	49/50	45/50
V. vulnificus	53/53	53/53	53/53	46/53	48/53
Vibrios and other bacteria	0/45	0/45	0/45	0/45	0/45

Table 2. Performance characteristics of the BAX Vibrio PCR Assay compared with conventional PCR, API20E and VITEK 2 Compact identification systems

Strain	Method	χ^2	Sensitivity	Specifity
Vibrio cholerae	BAX vs API 20E	1.33 (p=0.25)	48/48	1/4
	BAX vs VITEK Compact 2 GN Card	0 (p=1)	50/51	0/1
	BAX vs BAM PCR agarose	NA	NA	NA
	BAX vs BAM PCR Agilent 2100	NA	NA	NA
Vibrio parahaemolyticus	BAX vs API 20E	0 (p=1)	49/49	0/1
	BAX vs VITEK Compact 2 GN Card	3.2 (p=0.074)	45/45	0/5
	BAX vs BAM PCR agarose	0 (p=1)	49/49	0/1
	BAX vs BAM PCR Agilent 2100	0/0	50/50	0/0
Vibrio vulnificus	BAX vs API 20E	0.5 (p=0.48)	51/51	0/2
	BAX vs VITEK Compact 2 GN Card	2.25 (p=0.125)	49/49	0/4
	BAX vs BAM PCR agarose	0/0	53/53	0/0
	BAX vs BAM PCR Agilent 2100	0/0	53/53	0/0

χ^2 = McNemar's test with continuity correction (critical value = 3.84 for significant difference at p= 0.05)
N/A = not applicable
0/0 = indeterminable
Sensitivity = frequency + by BAX among isolates determined + by reference method
Specificity = frequency – by BAX among isolates determined – by reference method

Table 3. *Vibrio* isolates which were not correctly identified by one or more of the procedures; Good identification (green), poor identification (yellow) , incorrect identification or unidentified(red)

Isolate	BAX PCR	BAM PCR	BAM PCR Agilent 2100	API 20E Profile	API 20E	VITEK2 Compact Bionumber	VITEK2 Compact
Vc GCSL 3	+	+	+	1044125	*Aeromonas* spp. 79.4%	0425613151547211	Vc 95%
Vc GCSL 6	-	+	+	5146125	Vv 51.1% Vc 48.2%	0425613151506221	Vc 96%
Vc GCSL 15	+	+	+	5146125	Vv 51.1% Vc 48.2%	0425611151506211	Vc 96%
Vc GCSL 59	+	-	-	1046024	Vf 55.9%	1427311350501223	Unidentified Organism
Vp GCSL 4	+	+	+	4146107	Vp 99.9%	0025210150000001	*A. sorbia* 99%
Vp GCSL 22	+	+	+	4146107	Vp 99.9%	5025711340547221	Unidentified Organism
Vp GCSL 24	+	+	+	4144107	Vp 99.9%	5025711340547261	Unidentified Organism
Vp GCSL 25	+	-	+	4146107	Vp 99.9%	5025611340500262	Vp 97%
Vp GCSL 29	+	+	+	5146107	Vv 93.9%	5425711140500362	Vp 94%
Vp GCSL 50	+	+	+	4146107	Vp 99.9%	5025711140504221	Vv 97%
Vp GCSL 140	+	+	+	4146107	Vp 99.9%	5027711340547263	Unidentified Organism
Vv GCSL 12	+	+	+	5346125	Vc 88.8% Vv 10.7%	5225311140541201	Vv 99%
Vv GCSL 39	+	+	+	1246105	Vv 88.9%	5025611340500262	Vp 97%
Vv GCSL 48	+	+	+	5346105	Vv 98.9%	1421001100001000	*Sphingomonas paucimobilis* 95%
Vv GCSL 59	+	+	+	5146125	Vv 51.1% Vc 48.2%	5621711150501200	Vf 97%
Vv GCSL 245	+	+	+	0044004	*Grimontia hollisae* 62.2%	5021201140403201	*Sphingomonas paucimobilis* 89%

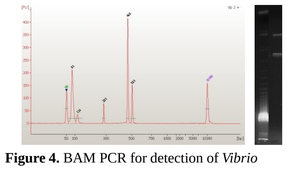

Figure 4. BAM PCR for detection of *Vibrio parahaemolyticus* (triplex assay for 450 bp fragment of *tlh* species specific marker, 500 bp fragment of *trh* virulence marker and a 270 bp fragment *tdh* virulence marker). Agilent 2100 (left) and gel electrophoresis (right)

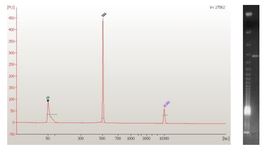

Figure 5. BAM PCR for detection of *Vibrio vulnificus*.(519 bp fragment of *vvh*), Agilent 2100 (left) and gel electrophoresis (right)

DISCUSSION

- The different groups of bacteria examined with the BAX *Vibrio* assay are shown in Table 1. Previously characterized bacterial isolates (200 strains) were tested. Correct identifications are shown for each of the methods except Vc where the BAM PCR identified a fragment of the cholera toxin gene. Of the 52 isolates examined, 16 were shown to carry that DNA fragment.
- None of the near neighbor and non-vibrio isolates were detected by the BAX assay, BAM methods or identified as Vc, Vp or Vv by the API20E and VITEK2 Compact.
- The sensitivity and specificity rates for the BAX PCR assay as compared with BAM conventional PCR for each of the pathogens with visualization of the PCR products on agarose gels and the Agilent 2100 Bioanalyzer are shown in Table 2. The performance characteristics of the BAX Vibrio assay are also compared with biochemical identification by the API20E and VITEK 2 Compact in Table 2.
- For Vc isolate identification, the BAX Vibrio qPCR assay was not statistically different from the API 20E or the VITEK. The assay was not compared with the BAM PCR as the BAM assay determines toxigenic Vc and not total Vc (Table 2).
- Both Vp and Vv isolates no statistical differences were shown between the BAX and the API20E, the BAX and the Vitek or the BAX and the BAM PCR (visualized on agarose gels or Agilent 2100 chips) (Table 2).
- Of the 52 Vc isolates tested, one (VcGCSL-6) was negative by the BAX assay. VcGCSL-6 was identified as Vc by the VITEK but Vv or Vc by the API20E as shown in Table 3. Four of the isolates were incorrectly identified by the API20E while one could not be identified by the VITEK and one isolate was poorly identified as Vc.
- For Vv, 53 isolates were evaluated and all were positive for Vv by both the BAX assay and the BAM PCR (on both agarose gels and the Agilent 2100 DNA chips). As shown in Table 3, ten isolates gave poor results with the API20E, while 5 were misidentified or poorly identified with the VITEK. The API profiles and VITEK Bionumbers identifications for each are listed.
- Fifty Vp isolates were evaluated with the BAX PCR and all were positive for the Vp target. The BAM conventional PCR also identified all 50. However, microfludic separation on the Agilent Bioanalyzer showed more of the amplified targets from the triplex Vp PCR assay than conventional gel electrophoresis (data not shown). The API20E identified 49/50 while the VITEK identified 45/50.

CONCLUSIONS

- The BAX Real time PCR assay for *Vibrio cholerae/parahaemolyticus/vulnificus* is a reliable and rapid alternative to the BAM methods for identification of Vc, Vv, and Vp isolates.
- The API 20E and VITEK 2 Compact systems gave accurate results for most of the isolates examined, however, some atypical Vc, Vp and Vv isolates were poorly identified or could not be identified even though alternative tests (BAM conventional biochemical screening tests, BAM conventional PCR or the BAX) identified the organisms correctly.
- The Agilent 2100 Bioanalyzer was shown to be more sensitive and accurate than gel electrophoresis for sizing and visualization of PCR fragments for the identification and characterization of pathogenic *Vibrio* spp.

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