Characterization of potentially pathogenic *Vibrio* spp. by *rpoB*-DHPLC

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Background

Rising sea water temperature due to global warming enhances the conditions for human pathogenic *Vibrio* spp. to grow and disperse even in temperate waters of the North and Baltic Sea. Because of the increased incidence of Vibrio infections in the last years, a rapid and accurate method is required to analyze and identify complex *Vibrio* spp. populations, specifically potential pathogenic Vibrio species, in environmental samples.

Research aim

Development of a new, fast, and cost efficient PCR-DHPLC protocol based on the *rpoB* gene for identification and separation of *Vibrio* spp. in environmental samples.

Material and Methods

To facilitate the identification of potential human-pathogenic species we designed Vibrio specific primers, amplifying fragments of 250-350 bp of the *rpoB* gene. Using the PCR products of different *Vibrio* species, we systematically improved the DHPLC conditions.

1. Primer Evaluation

2. *rpoB*-DHPLC

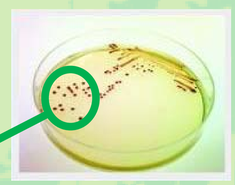
DNA extraction

Optimizing PCR conditions

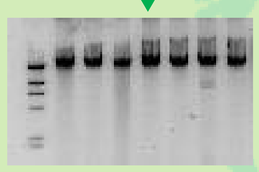
Amplification

Optimizing DHPLC conditions

3. Molecular comparison



Phenol-chloroform extraction



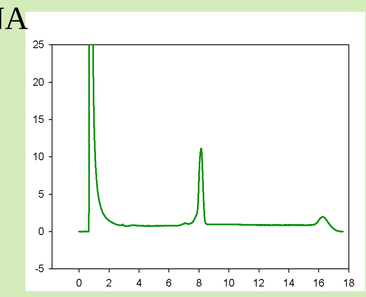
Genomic DNA on Agarose Gel

Amplification with Vibrio specific *rpoB*-Primers

PrV

PrV

Identification of Vibrio strains in the DHPLC analysis due to the individual running behavior of partial denatured DNA



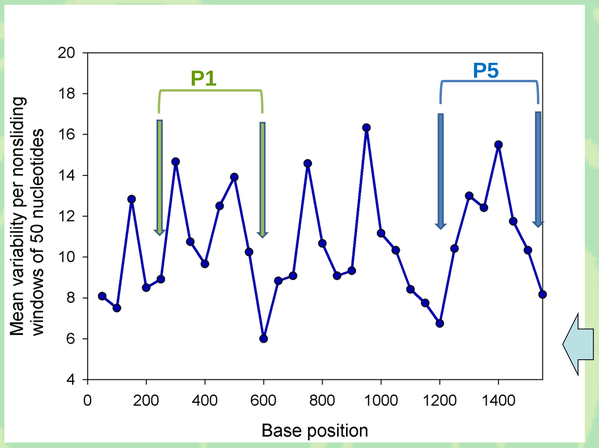
Optimizing DHPLC conditions:

- Comparison of PCR-products using *rpoB*-Primer with and without GC-clamp
- Adapting column temperature
- Adjustment of acetonitrile gradient by increasing the concentration of Buffer B (0.1 M TEAA with 25 % acetonitrile)

Results

Primer evaluation

- Selected Primer covering different regions of the targeted *rpoB*-gene were tested (Fig. 1)
- Vibrio specific primers fell into conserved regions of the *rpoB*-gene, while the targeted region is highly polymorphic (Fig. 2)
- **31** *Vibrio* species were tested using 5 Primer sets, **20-23** different *Vibrio* species were successfully amplified with PCR fragments of 250-350 bps



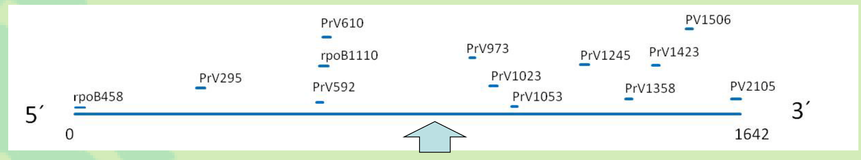


Figure 1: Partial *rpoB*-gene (1642 bp from 5´to 3´position) and primer binding sites. The *rpoB*-gene was amplified with primers rpoB458F, rpoB2105R and rpoB1110F according to Tarr *et* al. (2007) and Hazen *et* al. (2009).

Figure 2 Variable and conserved regions of *rpoB*-gene in *Vibrio* spp.. Mean variability based on the alignment of 24 Vibrio *rpoB* sequences per non-sliding windows of 50 nucleotides was calculated with the SVARAP-tool (Colson *et* al., 2006). Arrows point at primer binding sites of two primer-sets exemplarily.

DHPLC-application

- Adaptation of PCR protocol (i.e. attachment of a GC-clamp to 5'end of forward primers and a touchdown protocol) lead to more distinct and high absorbance DHPLC peak profiles (see arrow Fig. 3)

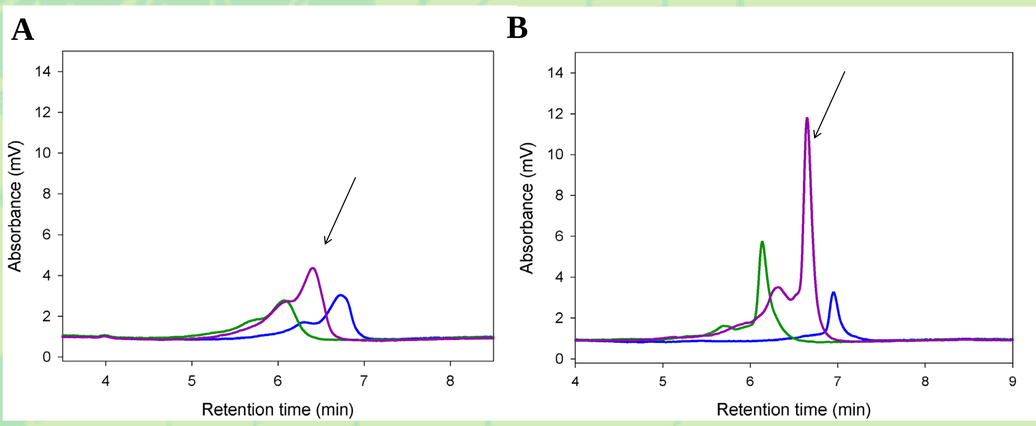


Figure 3: DHPLC analysis of 3 individual PCR-fragments using forward primer without (A) and with 40-bp GC-clamp (B). Arrows indicate increase in peak intensity after adaptation of the PCR protocol. Acetonitrile gradient was increased stepwise (45 % Buffer B for 0 min (loading), 49.8 % B for 0.5 min to 63.4 % B for 9.3 min at 62.5 °C (A) and 63.5 (B); flow rate 0.9 ml/min; injection volume 10 µl.

- By adjustment of temperature and acetonitrile gradient, we separated 6 *Vibrio* species according their retention behavior using Primer-set 5 (Fig. 4 a)
- Unique conditions of Primer-set 5: i.) short amplicon-length of 278 bp, ii.) two melting domains and iii.) relatively high GC-variability of amplicons
- We found a strong positive correlation between retention time and GC-content (Fig. 4 b)
- Closely related *Vibrio* species, e.g. *V. parahaemolyticus* and *V. alginolyticus* could not be separated due to their highly similar GC content (48,6% and 48,7%)

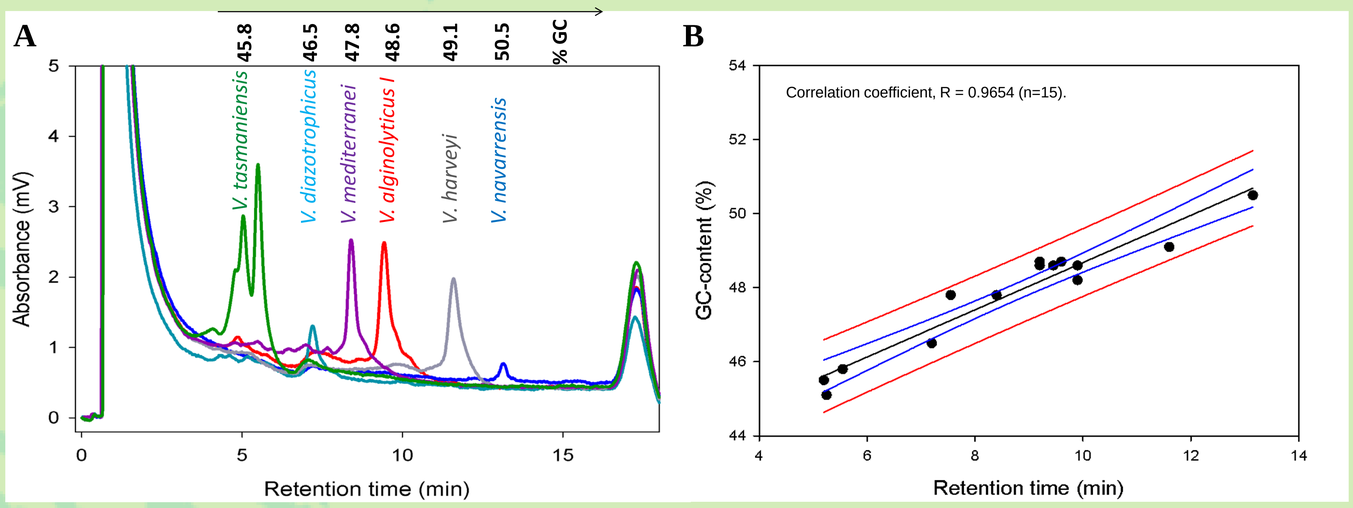


Figure 4: DHPLC analysis of individual PCR-products of 6 Vibrio species amplified with primer-set 5 (with GC-clamp). Acetonitrile gradient: 45 % Buffer B for 0 min (loading), 54 % B for 2 min to 60 % B for 14 min at 63.5 °C; flow rate 0.9 ml/min; injection volume 10 µl (A). Retention time observed in the DHPLC analysis was correlated with GC-content of the *rpoB*-sequences (B).

Conclusion and Outlook

- The designed primer-sets are specific for the genus Vibrio and include highly variable regions of the *rpoB*-gene
- The *rpoB*-DHPLC assay is a sensitive tool to differentiate between *Vibrio* species, but is not applicable for separation of vibrios with similar GC content
- For successful discrimination of PCR-fragments, incorporation of a GC-clamp, adaption of column temperature and acetonitrile gradient were essential
- In addition to the mean variability in the *rpoB*-gene, other discriminative factors play a crucial role for separation in the DHPLC-system:
  - ➢ Optimal separation conditions on sequence basis are: i.) high GC-variability of amplicons with a threshold > 0.5%, ii.) short amplicon-length and iii.) different melting-domains
- **Ongoing research:** Optimizing Vibrio specific primers on the basis of the *rpoB*-gene, evaluation of primer-sets in DHPLC-system and test the new assay for mixed and environmental samples

Sources: Colson P, Tamalet C, Raoult D (2006) SVARAP and aSVARAP: simple tools for quantitative analysis of nucleotide and amino acid variability and primer selection for clinical microbiology. *BMC Microbiology* **6:**21

Hazen TH, Kennedy K, Chen S, Yi S & Sobecky PA (2009) Inactivation of mismatch repair increases the diversity of *Vibrio paraheamolyticus. Environ Microbial.* **11:** 1254-1266

Tarr C, Patel J, Puhr N, Sowers E, Bopp C & Strockbine N (2007) Identification of *Vibrio* isolates by a multiplex PCR assay and *rpoB* sequence determination. *J Clin Microbiol* **45**: 134-140