

RESEARCH ARTICLE

gbpA as a Novel qPCR Target for the Species-Specific Detection of *Vibrio cholerae* O1, O139, Non-O1/Non-O139 in Environmental, Stool, and Historical Continuous Plankton Recorder Samples

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Abstract

The *Vibrio cholerae* N-acetyl glucosamine-binding protein A (GbpA) is a chitin-binding protein involved in *V. cholerae* attachment to environmental chitin surfaces and human intestinal cells. We previously investigated the distribution and genetic variations of *gbpA* in a large collection of *V. cholerae* strains and found that the gene is consistently present and highly conserved in this species. Primers and probe were designed from the *gbpA* sequence of *V. cholerae* and a new Taq-based qPCR protocol was developed for diagnostic detection and quantification of the bacterium in environmental and stool samples. In addition, the positions of primers targeting the *gbpA* gene region were selected to obtain a short amplified fragment of 206 bp and the protocol was optimized for the analysis of formalin-fixed samples, such as historical Continuous Plankton Recorder (CPR) samples. Overall, the method is sensitive (50 gene copies), highly specific for *V. cholerae* and failed to amplify strains of the closely-related species *Vibrio mimicus*. The sensitivity of the assay applied to environmental and stool samples spiked with *V. cholerae* ATCC 39315 was comparable to that of pure cultures and was of 10² genomic units/l for drinking and seawater samples, 10¹ genomic units/g for sediment and 10² genomic units/g for bivalve and stool samples. The method also performs well when tested on artificially formalin-fixed and degraded genomic samples and was able to amplify *V. cholerae* DNA in historical CPR samples, the earliest of which date back to August 1966. The detection of *V. cholerae* in CPR samples collected in cholera endemic areas such as the Benguela Current Large Marine Ecosystem (BCLME) is of particular significance and represents a proof of concept for the possible use of the CPR technology and the developed qPCR assay in cholera studies.

design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction

Vibrio cholerae is the causative agent of cholera, an enteric disease which affects the intestinal tract, characterized by severe diarrhea, vomiting and dehydration. Cholera continues to be a major cause of morbidity and mortality around the world as every year 3–5 million people are infected with cholera and 100,000–120,000 people die from the disease [1].

V. cholerae is an environmental bacterium which thrives in brackish and estuarine water around the world mostly in association with a variety of environmental reservoirs and/or hosts such as plankton, bivalves, other aquatic animals and plants, and aquatic sediments [2]. The role of these substrates in cholera endemicity and/or the transmission of the disease to humans is well documented [2,3] and monitoring the bacterium in environmental sources is of utmost importance to understand its ecology and spread, and to take preventive measures for its control [4].

Culture-dependent methods used to detect and classify *V. cholerae* are laborious and time-consuming [5] as they require prolonged incubation and growth on selective media to reduce the number of non-target organisms [6, 7]. Alternative, more rapid, specific and sensitive molecular-based techniques have been developed recently. The added value of molecular procedures is the capability to detect *V. cholerae* in DNA extracted from environmental samples, also when present in the viable but not culturable (VBNC) form, a dormant state that allows bacteria to survive and persist in the natural environment under unfavorable conditions [8,9].

Among the techniques reported in the literature some procedures have been developed for *V. cholerae/V. mimicus* clade detection [10, 11], with *V. mimicus* phylogenetically being the most closely related species to *V. cholerae*, while other methods are multiplex polymerase chain reaction assays for simultaneous detection of *V. cholerae* and other pathogens [12,13]. However, most of these analysis procedures are non-quantitative [14] or were only tested on few types of samples (mainly pure culture, stool or water samples) [15–20].

Generally, direct PCR-based analyses of complex environmental samples are problematic due to the low level of *V. cholerae* in environmental matrices, the concomitant large number of non-target microorganisms and the presence of PCR inhibitors [21]. As a consequence, there is an obvious lack of a rapid, sensitive, specific and quantitative method to detect *V. cholerae* species in these samples. This particularly holds true for detection of the bacterium in highly problematic samples such as formalin-fixed samples where DNA can be damaged (e.g. fragmented) and the PCR reaction can be hampered by the presence of inhibitors [22]. These types of samples such as natural history collections and/or other repository collections from public and private institutions worldwide are of outstanding value to a wide range of studies, including genetic, evolutionary, biogeographic, ecological and epidemiological studies, especially with the recent development of molecular biology techniques [23,24]. For instance, insights into the ecology of vibrios in coastal marine environments have recently been obtained by the retrospective analysis of formalin-fixed plankton samples collected in the last 60 years by the Continuous Plankton Recorder (CPR) Survey [25].

To provide a method for the detection and quantification of *V. cholerae* in problematic samples we identified a new taxonomic marker, namely the *gbpA* gene encoding for the N-Acetylglucosamine (GlcNAc) binding protein A of *V. cholerae*. GbpA is a 53 kDa extracellular secreted and surface-associated protein that has recently been shown to be involved in *V. cholerae* attachment to environmental chitin surfaces and cultured intestinal epithelial cells via the same GlcNAc binding specificity [26–28]. In a previous study we evaluated the distribution of the gene in 488 *V. cholerae* strains of environmental and clinical origin, belonging to different serogroups and biotypes and found that *gbpA* is always present in *V. cholerae* [28]. In addition, *in silico* analysis performed on full sequences of the gene in different *Vibrio* species revealed

that *gbpA* differs substantially within the *Vibrio* genus whilst it is highly conserved within the species *V. cholerae* [28].

As a follow up to our previous observations, this study developed a new, rapid, sensitive and quantitative species-specific Taq-Man based Real-Time PCR protocol targeting the *gbpA* gene of *V. cholerae*. The protocol is optimized for capillary PCR and works efficiently for the detection of the bacterium in problematic environmental samples. In addition, being based on the amplification of a 206 bp small DNA fragment, the developed protocol is suitable for the detection of *V. cholerae* in highly damaged samples such as historical formalin-fixed CPR samples.

Materials and Methods

Ethics Statement

For the collection of freshwater and marine sediment samples local competent authorities (Municipality of Goro and Municipality of Genoa) were informed and allowed the sampling for research purpose only. For the collection and use of the bivalve *Mytilus galloprovincialis* no specific permits were required according to Italian legislative decree 4 March 2014, n. 26 and EU directive 2010/63/UE (Legislation for the protection of animals used for scientific purposes). In any case, we declare that the present study did not involve endangered or protected species and that stress was minimized during both animal samplings and experiments. Mussels were purchased from an aquaculture farm (Arborea, Italy) and sacrificed in the laboratory by laboratory staff for the purpose of the study. To ameliorate the suffering of the animals the method of sacrifice consisted in the cutting of the posterior adductor muscle followed by rapid freezing of tissues (this practice is known to ameliorate the suffering of the bivalve without the use of anesthetics/analgesics which are not recommended for this type of study).

For the collection and use of fecal samples written informed consent was obtained from the parents of a healthy 2-years old male donor according to the Italian law. All work with fecal human samples was approved by the competent ethics committee (Sezione 1, Comitato Etico Regione Liguria) on 09/12/2014, approval number 501REG2014.

Bacterial strains, culture conditions and DNA extraction

A total of 129 bacterial strains belonging to different *Vibrio* and other related species were used in this study (S1 Table). Strain *V. cholerae* O1 El Tor N16961 (ATCC 39315) was used as the reference strain. Bacterial cells were grown in Luria Bertani (LB: yeast 5 g/l, bacto-tryptone 10 g/l, NaCl 10 g/l) broth or agar, and on Thiosulfate-citrate-bile salts-sucrose TCBS (Lab M, United Kingdom) agar at 37°C. After overnight growth, cells were harvested by centrifugation (4500 g for 10 min), washed twice with phosphate-buffered saline (PBS: NaCl 8 g/l; KCl 0.2 g/l; Na₂HPO₄·12H₂O 3.62 g/l; KHPO₄ 0.24 g/l) or artificial sea water (ASW: NaF 1.9 mg/l; SrCl₂·6H₂O 13 mg/l; H₃BO₃ 20 mg/l; KBr 67 mg/l; KCl 466 mg/l; CaCl₂·2H₂O 733 mg/l; Na₂SO₄ 2.66 g/l; MgCl₂·6H₂O 3.33 g/l; NaHCO₃ 133 mg/l; NaCl 27.65 g/l; pH 8) and resuspended in the appropriate buffer, according to the assay to be performed, to reach a final concentration of 0.2 OD₆₀₀. For each strain, DNA was extracted from pure culture by using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Milan, Italy) according to the manufacturer's instructions.

PCR primers and probe development

The primers pairs: *gbpA*-seq1F (5-tca ctc tga act gcg tct gg-3) and *gbpA*-seq1R (5-ttg gtt agc gtc tca gag tca a-3) and *gbpA*-seq2F (5-cac tcg cgt gtt tga taa cg-3) and *gbpA*-seq2R (5-gtg gag agg tag cca ctg ga-3) amplifying positions 1–767 and 690–1458 of the *gbpA* gene of *Vibrio*

cholerae N16961 (GenBank accession number: EU072441.1) respectively, were previously designed to amplify the entire *gbpA* open reading frame (ORF) in 36 representative *V. cholerae* strains [28]. Amplified fragments from the PCR reaction were purified with the High Pure PCR Template Preparation Kit (Roche Diagnostics, Milan, Italy) and sequenced by using the Sanger dye-terminator method [28]. The *gbpA* gene sequences were then aligned using the BIOEDIT software [29] and primers and a probe were designed to target a species-specific region of the gene showing the lowest sequence variability (>99%) within *V. cholerae* using Primer3 [30, 31]. The chosen primer and probe sequences for this qPCR assay were Vc *gbpA* F (5-ccg cag ctt cct tct aca ac-3), V.c *gbpA* R (5-ggc ttt ggt tag cgt ctc ag-3) and V.c *gbpA* pr (5-FAM-aac cca gca ggt caa atc att cca agt a-BBQ) probe. The Nucleotide Basic Local Alignment Search Tool (BLASTn) and Primer-BLAST tool [32] were used to check that the Vc *gbpA* F, V.c *gbpA* R and V.c *gbpA* pr did not match any non-target *Vibrio* species. The primers and probes were synthesized by Tib Molbiol srl (Genoa, Italy).

Construction of standard curves for qPCR and Interference assays

To determine the limits of detection, linear ranges, and amplification efficiencies of the qPCR assay, a standard curve, based on accurately quantified genomic DNA of *V. cholerae* ATCC 39315 strain (Genetic PCR solution TM, Alicante, Spain), was constructed. The genomic standard (GS) was purified and checked for the absence of PCR inhibitors by constructing a standard curve plot (inhibition plot). The concentration was determined by fluorometry and expressed as genomic units/ μ l (GU/ μ l). GS was serially diluted to prepare solutions containing 10^6 to 10^1 genome copies per reaction (GU/rx). For each qPCR assay, a total of three separate runs were performed (each run in triplicate) to determine intra-assay and inter-assay reproducibility (assessed by computing the coefficient of variation, %CV). The limit of detection of the qPCR assay was determined as the lowest concentration at which 100% of the replicates were detected. To assess interference from non-target DNA, increasing concentrations of purified genomic DNA of *V. cholerae* ATCC 39315 (from 10^1 to 10^4 GU/ μ l) were also quantified by the qPCR in the presence of purified DNA from *V. mimicus* UM 6812 that was added to the qPCR reaction mixture at a concentration of 0.5 ng/rx (corresponding to 8×10^4 GU/rx).

qPCR amplification

The qPCR amplification protocol was set up on a Light Cycler 1.5 instrument (Roche Diagnostics, Milan, Italy) using a Light Cycler TaqMan Master Mix chemistry [33]. Amplification reaction mixtures (20 μ l) contained: 1x TaqMan Master Mix; 200 nM primers; 25 nM probe; DNA sample (0.2–2 ng/ μ l) and five microlitres of DNA template. The PCR program used was as follows: initial denaturation at 95°C for 10 min, subsequent 45 cycles of denaturation at 95°C for 10 s, annealing at 59°C for 20 s and elongation at 72°C for 1 s, followed by a cooling step at 40°C for 30 s. Amplicons were visualized by agarose gel electrophoresis using ethidium bromide solution. Accurately quantified genomic DNA of *V. cholerae* ATCC 39315 strain was used as a standard (Genetic PCR solution, Alicante, Spain). For quantification, the log of the number of genome units (GU) of a dilution series of the sample was plotted versus the cycle number at which the fluorescent signal increased above threshold (C_q-value).

qPCR studies with artificially spiked environmental and stool samples

Drinking and seawater samples. One litre of tap water collected at the laboratory of microbiology of the University of Genoa and sea surface water (ASW) obtained by reconstituting Sea Salts (Sigma—Aldrich) with demineralized hypo-osmolar water to 30‰ final concentration was spiked with different concentrations of *V. cholerae* ATCC 39315 (from 10^6 to 10^3 cells

per litre) and subsequently filtered onto 0.22 µm-pore-size Millipore membrane (47 mm in diameter) (Millipore, Milan, Italy). DNA from filter-bound material was extracted by using Rapid Water DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions.

Aquatic sediment samples. Aliquots of 1 g of freshwater (Brugneto Lake, Genoa, Italy) and marine sediment (Sacca di Goro, Ferrara, Italy) were spiked with different concentrations of *V. cholerae* ATCC 39315 (from 10^6 to 10^1 cells per gram). The absence of *V. cholerae* in these samples was previously evaluated and confirmed by qPCR. A total of 0.5 g from each sample was used for DNA extraction by using Ultra Clean soil DNA kit (MoBio Laboratories, Solana Beach, CA, USA) according to manufacturer's instructions.

Bivalve samples. Freshly harvested mussels (*Mytilus galloprovincialis*) obtained from a fishfarm in Sardinia (Mediterranean Sea, Italy) were transported on ice to the Laboratory of Microbiology of the University of Genoa, stored at refrigeration temperature (5°C), and subjected to microbiological analysis within 4 to 8 h. The absence of *V. cholerae* in bivalve samples was previously evaluated and confirmed by qPCR. Aliquots of 1 g of *V. cholerae*-free mussel flesh homogenate were then spiked with different concentrations of *V. cholerae* ATCC 39315 (from 10^6 to 10^1 cells per gram). A total of 0.25 g from each sample was used for DNA extraction by using High Pure PCR Template Preparation Kit (Roche Diagnostics, Milan, Italy) according to manufacturer's instructions.

Stool samples. A single fecal sample (1 g) was obtained from a healthy 2-years old male donor. The sample was spiked with different concentrations of *V. cholerae* ATCC 39315 (from 10^6 to 10^1 cells per gram). A total of 0.5 g from each sample was used for DNA extraction by using Ultra Clean soil DNA kit (MoBio Laboratories, Solana Beach, CA, USA) according to manufacturer's instructions.

For the construction of standard curves, each DNA sample from the different above mentioned preparations was run in triplicate by the qPCR method.

qPCR studies with artificial formalin-fixed samples

V. cholerae O1 El Tor (ATCC 39315) was grown in Luria Bertani broth as previously described and resuspended in PBS to reach a final concentration of 10^8 CFU/ml. 10ml of the suspension was then spiked with 400 µl of neutral buffered Formaldehyde (4% final concentration) and serially diluted to prepare solutions containing 10^6 to 10^1 GU/rx. Genomic DNA was extracted after 1, 2, 4 and 8 weeks using the protocol reported in Vezzulli *et al.* [25] for the analysis of CPR samples (see below). For the construction of standard curves, each DNA sample was run in triplicate by the qPCR method.

qPCR studies with artificially fragmented DNA samples

Genomic DNA was extracted from pure culture of *V. cholerae* O1 El Tor (ATCC 39315) as previously described. 1.2 µg of genomic DNA was artificially fragmented using a Proven Covaris AFA shearing process (Covaris Ltd, Brighton UK) generating an homogenous pool of fragments having an average size of 276 bp. Fragmented genomic DNA samples were purified using an Agencourt AMPure XP system (Beckman Coulter s.r.l. Milan, Italy) following the manufacturer's instructions and run on an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA kit (Agilent Technologies). Each DNA sample was then run in triplicate by the qPCR method.

For comparison, the same samples were also analyzed by the qPCR protocol described by Chun *et al.* [15] targeting the 16S-23S rRNA Intergenic Spacer Regions (ISR) for the detection and quantification of *V. cholerae*. The Promega GoTaq qPCR Master Mix kit, optimized for

use with glass capillaries and containing a hot start polymerase, was used as the master mix base for all reactions. Primers used were: prVC-F 5-tta agc gtt ttc gct gag aat g-3 and prVC-R 5 agt cac tta acc ata caa ccc g-3 [15]. Each reaction mixture contained 0.2 μmol of each primer in a final volume of 20 μl . The PCR programme used was as follows: initial denaturation at 95°C for 2 min, subsequent 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s and elongation at 72°C for 45 s, followed by final elongation at 72°C for 30 s. PCR runs were analyzed directly in the LightCycler using melting analysis and the analysis software provided with the instrument.

qPCR studies with historical formalin-fixed samples (CPR samples)

Formalin-fixed plankton samples collected in different coastal areas in the last 60 years by the Continuous Plankton Recorder (CPR) Survey were retrieved from the CPR archive in Plymouth (England). The CPR is a high-speed plankton sampler designed to be towed from commercially operated ships of opportunity over long distances [34]. Sampling takes place in the surface layer (7 m) and plankton is collected on a band of silk (mesh size 270 μm) that moves across the sampling aperture at a rate proportional to the speed of the towing ship. On return to the laboratory, the silk is removed from the device and divided into individual samples that are stored in airtight plastic boxes in buffered formalin (usually comprising 4–10% buffered formalin) [36].

To test the performance of the qPCR method to detect *V. cholerae* O1, O139, Non-O1, and Non-O139 in historical formalin-fixed samples three different sets of CPR samples were used. The first set included two samples: samples 413R and 485R collected in the North Sea off the river Rhine estuary in 1998 and 2004, respectively (S2 Table). Both of these samples were previously analysed by 16SrDNA amplicon pyrosequencing, revealing the presence of read sequences showing >95% identity to *V. cholerae* [25]. A second set included 5 samples collected in the early years of the CPR Survey in different coastal areas of the North Sea and North Atlantic Ocean: 2EB collected off Nova Scotia in 1961; 11IN2 collected in the Irish Sea in 1966; 228A collected near the Shetland Islands in 1966; 157SB collected in the Bay of Biscay in 1971 and 157SB collected off the Iberian Coast in 1971 (S2 Table). Finally, a third set included 18 samples collected by the South African CPR sister survey in the Benguela Current Large Marine Ecosystem (BCLME) region during the inaugural BC-CPR survey (Luanda, Angola—Durban, South Africa) in September 2011: 4CT 2, 4CT 4, 4CT 6, 4CT 24, 4CT 26, 4CT 28, 4CT 38, 4CT 40, 4CTend, 6CT 18, 6CT 20, 7CT 20, 7CT 22, 7CTend, 8CT 26, 8CT 28, 8CT 30, 8CT 32 (S2 Table).

Genomic DNA was extracted from CPR samples using the protocol previously described in Vezzulli *et al.* [25]. Briefly, for each sample, the filtering silk was cut into five replicate (1 cm^2) sections. Each section was placed in a sterile tube, after which 25 ml of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) were added and vortexed to detach plankton from the silk mesh. Samples were left to rest at room temperature for 24 h; the plankton suspension was then gently centrifuged (400g) and the pellet transferred to a sterile microcentrifuge tube. Fifty μl of lysozyme (2 mg/ml in 10 mM Tris-HCl, pH 8.0) was added to the sample that was then vortexed vigorously for 1 min. After the addition of 180 μl 10% sodium dodecyl sulphate and 25 μl proteinase K (10 mg/ml), the sample was vortexed for 30 sec. The sample was then incubated at 56°C for 1 h, heated at 90°C for 1 h in a dry-block heater, vortexed for 10 sec and centrifuged at 12000 g for 3 min. After addition of 200 μl guanidine hydro-chloride lysis solution and 200 μl ethanol, the sample was centrifuged (12000 g for 10 sec). The supernatant was then transferred to QIAamp MinElute column (Qiagen, Valencia, CA, USA) and processed according to the manufacturer's recommendation. The retained DNA was purified with QIAquick PCR

purification columns (Qiagen spa, Milano, Italy) up to a final yield of 1–7 mg/ml. The LightMix Modular PhHV spiked Extraction Control (Roche Diagnostics, Milan, Italy) was used to test for the presence of DNA inhibitors in representative CPR samples (see below). Each DNA sample was run in triplicate by the qPCR method. A “*Vibrio* relative abundance index” (VAI), defined as the ratio of *Vibrio* spp. cells to the total number of bacterial cells assessed by real-time PCR using genus-specific (Vib1 5-ggc gta aag cgc atg cag gt-3 and Vib2 5-gaa att cta ccc ccc tct aca g-3) [35] and bacterial universal primers (967f 5-caa cgc gaa gaa cct tac c-3 and 1046r 5-cga cag cca tgc anc acc-3 [36] respectively, was also measured on the 18 samples collected in southern Africa in 2011 following the protocol previously described in Vezzulli *et al.* [25].

Results and Discussion

Quality evaluation of *V. cholerae* primers and Taq-Man probe

Species-specific PCR primers (VcgbpA F; VcgbpA R) and probe (VcgbpA pr) for the detection and quantification of *V. cholerae* were designed within the region of the *gfpA* gene showing the lowest sequence variability in *V. cholerae* species according to bioinformatics analysis. The primers target a small DNA fragment (206 bp) amplifying positions 566–771 of the *gfpA* gene of *V. cholerae* O1 El Tor (ATCC 39315). The quality of the developed primers and probe was evaluated in terms of *in-silico* target species coverage (sensitivity) and specificity against the GenBank NIH genetic sequence database [37], using BLASTn and the Primer-BLAST tool [32]. The primers and probe cover almost 100% of *V. cholerae* target sequences within the reference database and showed a good performance in term of specificity as BLAST results did not show any significant homology to other published sequences in the GenBank, DDBJ, and EMBL databases. Interestingly, *V. mimicus*, a highly related species to *V. cholerae*, was not completely matched by the primers and probe.

Evaluation of *V. cholerae* qPCR specificity with pure cultures

A first set of trial qPCR experiments was conducted on genomic DNA extracted from pure culture of *V. cholerae* ATCC 39315 for setting optimal annealing temperatures and primer concentrations (corresponding to the highest qPCR specificity without reduction in yield) that were determined to be 59°C and 200 nM, respectively. qPCR specificity was evaluated by testing the developed primers and protocol on genomic DNA purified from 79 *V. cholerae* strains of environmental and clinical origin, belonging to different serogroups and biotypes, and a total of 48 strains belonging to other *Vibrio* species (4 *V. aestuarianus*, 4 *V. alginolyticus*, 1 *V. anguillarum*, 2 *V. corallilyticus*, 3 *V. harveyi*, 1 *V. metecus*, 2 *V. mimicus*, 20 *V. parahaemolyticus*, 1 *V. parilis*, 5 *V. splendidus*, 2 *V. tapetis*, 2 *V. vulnificus*, 1 *Vibrio* vent) (S1 Table). Strains *Escherichia coli* CECT4076 and *Salmonella enterica* Typhi CECT409 were also included in the analysis (S1 Table).

Results showed that all *V. cholerae* strains were efficiently identified by the qPCR (Cq-values ranging 22–23 at 10^5 GU/rx) while all other *Vibrio* species showed negative Cq-values (Cq-values over 40 were considered negative). Electrophoresis analysis on 2% agarose gels stained with ethidium bromide confirmed the absence of non-specific products or primer dimers.

To assess if the method is affected by interference from non-target DNA, a condition commonly found in direct PCR analysis of complex DNA samples, proportional concentrations of *V. cholerae* genomic DNA (from 10^1 to 10^4 GU/ μ l) were tested in the presence of purified DNA from *V. mimicus* UM 6812 that was added to the qPCR reaction mixture at a concentration of 0.5 ng/rx (corresponding to 8×10^4 GU/rx). Results showed that the qPCR protocol specifically detected target DNA also in the presence of *V. mimicus*. *V. cholerae* DNA was detected at similar Cq-values to those observed when performing the reaction in the absence of non-

Table 1. qPCR detection of *V. cholerae* ATCC 39315 cells in mixed cultures containing *V. mimicus* UM 6812.

<i>V. cholerae</i> concentration	Cq
10 ⁴ GU/μl	26.15±0.35
10 ⁴ GU /μl+ <i>V. mimicus</i> DNA	26.15±0.49
10 ³ GU /μl	29.35±0.21
10 ³ GU /μl+ <i>V. mimicus</i> DNA	29.40±0.42
10 ² GU /μl	32.95±1.06
10 ² GU /μl+ <i>V. mimicus</i> DNA	32.90±0.14
10 ¹ GU /μl	36.9±0.71
10 ¹ GU /μl+ <i>V. mimicus</i> DNA	36.75±0.35

qPCR detection of *V. cholerae* ATCC 39315 cells in mixed cultures containing 0.5 ng/rx (corresponding to 8x10⁴ GU/rx) of *V. mimicus* UM 6812. Quantification cycle (Cq) is expressed as mean ± standard deviation calculated from two spiking experiments each quantified in triplicate on the same run (n = 6)

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target DNA (Table 1). Moreover Cq-values were positively correlated with *V. cholerae* proportions (R² = 0.98), indicating that non-target DNA did not interfere with the *gbpA* gene amplification. These results appear to be of particular relevance if we consider that *V. mimicus* is phylogenetically the most closely related species to *V. cholerae*, having been previously considered as a biotype of this species [38–40]. The protocol we developed not only discriminates between the two closely related species but is also not affected by interference of closely related DNA, suggesting its potential valuable use in complex samples (e.g. environmental samples) where DNA of the target organism would likely be present amongst a large number of different microbial cells.

Standard curves and limit of detection with pure cultures

Standard curves for the qPCR protocol were generated by preparing serial ten-fold dilutions of known concentrations (10⁶–10⁰ GU/rx) of *V. cholerae* ATCC 39315 genomic standard DNA (Fig 1). The amplification efficiencies of the qPCR assays were in the range of 82–116% and were able to detect the *gbpA* gene down to levels of 50 gene copies (50 genome equivalents considering that a single copy of the *gbpA* gene is present in the *V. cholerae* genome) (Fig 1).

Linearity was optimal (R² > 0.99) over a 6-log-unit dynamic range with a coefficient (calculated on genome copy values) of intra-assay variation of < 5% and the inter-assay variation of < 15%.

Performance of *V. cholerae* qPCR in environmental and stool samples

The sensitivity of the method and amplification efficiency were evaluated on DNA extracted from different environmental matrices (drinking water, sea water, freshwater sediment, marine sediment, bivalve flesh) and stool samples spiked with known concentrations of *V. cholerae* ATCC 39315 cells (10⁶–10³ cells/l for drinking and sea surface water; 10⁶–10¹ cells/g for all other matrices). Quantitative amplification parameters of the performed assays are reported in Table 2. Linearity for all the assays was satisfactory (R² > 0.98) over a 5-log-unit dynamic range and the overall PCR amplification efficiencies ranged from 0.89 to 1.28. Detection limits corresponding to the smallest amount of template DNA resulting in positive amplification were of 10² GU/l for drinking water and sea surface water, 10¹ GU/g for freshwater and marine sediments and 10² GU/g for mussel flesh and stool samples (Table 2). Generally, it was found that the qPCR performance (e.g. efficiency and sensitivity) in DNA extracted from seawater

V. cholerae ATCC 3915

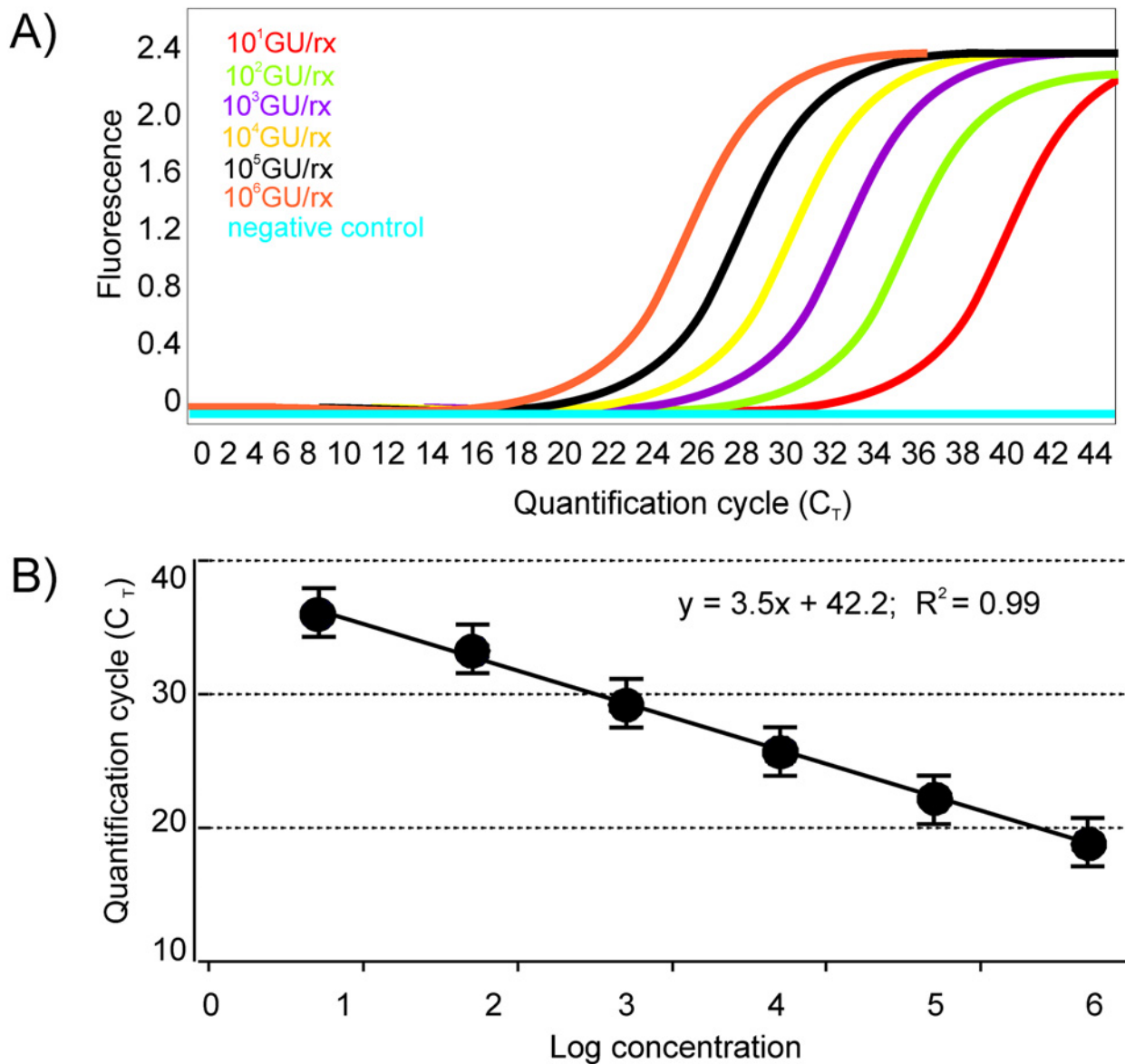


Fig 1. Sensitivity of the qPCR assay for detection of *V. cholerae* O1 El Tor N16961. DNA was amplified with the gbpA TaqMan primers in the presence of the gbpA fluorogenic probe. (A) Amplification plot of *V. cholerae* ATCC 39315 sample dilutions containing 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 genome copies per reaction (GU/rx). (B) Plot of mean C_T -values from three replicates tested against the *V. cholerae* DNA inputs. Error bars indicate the standard deviations of the means.

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and drinking water samples was similar to that obtained with DNA extracted from a pure bacterial culture, thus showing no inhibition of the amplification. In contrast, the performance of the qPCR assay was slightly lower, but overall good, in sediment samples where PCR amplification can be severely hampered by the presence of inhibitory substances, which are co-extracted with nucleic acids, such as humic acids, other organic polymers and clay particles [33, 41]. Aquatic sediments are putative environmental reservoirs for *V. cholerae* as it was recently

Table 2. Amplification parameters for the qPCR assay applied to environmental and stool samples.

Sample	Efficiency	Slope	Y intercept	R ²	Linear range	Detection limit
Drinking water	100±11	-2.91±0.14	41.1±0.5	>0,98	10 ⁶ –10 ¹ GU/L	10 ² GU/L
Sea water	100±15	-3.02±0.16	40.9±0.6	>0,98	10 ⁶ –10 ¹ GU/L	10 ² GU/L
Freshwater sediment	113±5	-3.06±0.12	38.7±0.5	>0,99	10 ⁶ –10 ¹ GU/gr	10 ¹ GU/g
Marine sediment	112±12	-3.07±0.18	38.9±0.4	>0,99	10 ⁶ –10 ¹ GU/gr	10 ¹ GU/g
Mussels flesh	105±9	-3.21±0.14	46.9±0.4	>0,99	10 ⁶ –10 ¹ GU/gr	10 ² GU/g
Stool	83±13	-3.82±0.12	46.9±0.7	>0,99	10 ⁶ –10 ¹ GU/gr	10 ² GU/g

The values of Efficiency, Slope and Intercept are expressed as mean ± standard deviation calculated from two spiking experiments with *V. cholerae* ATCC 39315 cells, each quantified in triplicate on the same run (n = 6)

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shown that *Vibrio* bacteria, including the species *V. cholerae*, can be found at concentrations up to an order of magnitude higher in sediment than in seawater [42]. Although, to date, the role played by aquatic sediments in the persistence and spreading of toxigenic *V. cholerae* is not entirely clear, research must rely on methods able to provide rapid and quantitative estimates of the bacteria in this problem area. The qPCR protocol developed in this study shows a good performance and can be employed for detection and quantification of *V. cholerae* cells in aquatic sediment samples.

In mussel flesh the sensitivity of the qPCR showed a detection limit (10² GU/g), around one log—unit lower than values obtained for the sediment but similar to limits reported in other studies obtained with a qPCR assay for endpoint detection [16]. Again this can be dependent upon the presence of PCR inhibitors in these samples. The qPCR assay may therefore be considered a useful tool for rapid and specific detection of *V. cholerae* in harvested and post-harvested bivalve molluscs.

The same sensitivity value of 10² GU/g was also observed when performing the analysis on stool samples. Because the abundance of *V. cholerae* cells in cholera-affected humans is normally ranging from 10⁷ to 10⁸ CFU/g and up to 10¹¹ to 10¹³ CFU/g or more in case of severe cholera [3], it can be inferred that the qPCR developed in this study should be also sufficiently sensitive to directly detect *V. cholerae* from diarrheal stool specimens. Furthermore, considering that the *V. cholerae* load in stools from convalescent and long-term carriers is reportedly in the range of 10² to 10³ CFU/g [43, 44] the method is also valuable for the bacterium detection in these samples.

It has finally to be considered that, apart from CPR samples (see next paragraph), all experiments in this study are based on artificially spiked samples. This means that for matrices like animal tissues, stool or environmental samples, the detection limit might be different to artificially spiked samples (e.g. depending upon the different extraction efficiency, presence of non-target DNA, inhibitors, etc). For such problematic samples the use of the LightMix Modular PhHV spiked Extraction Control specifically designed for the LightCycler may be considered as a spiked internal control in a further development of the current qPCR protocol.

Performance of *V. cholerae* qPCR in formalin-fixed and degraded samples

Fixation with formalin, a 37–40% (w/v) of formaldehyde gas in water, has traditionally been used for the fixation of biological and environmental samples for subsequent microscopic analyses and other studies.

Table 3. Amplification parameters for the qPCR assay applied to artificial formalin-fixed samples.

Sample	Efficiency	Slope	Y intercept	R ²	Linear range	Detection limit
ASW	104±6	-3.22±0.12	41.4±0.4	>0,99	10 ⁶ –10 ¹ GU/rx	10 ¹ GU/rx
4% formalin 1 week	94±9	-3.46±0.12	41.9±0.4	>0,99	10 ⁶ –10 ¹ GU/rx	10 ¹ GU/rx
4% formalin 2 week	96±7	-3.43±0.08	42.3±0.8	>0,99	10 ⁶ –10 ¹ GU/rx	10 ¹ GU/rx
4% formalin 4 week	94±6	-3.47±0.13	43.4±0.3	>0,99	10 ⁶ –10 ¹ GU/rx	10 ² GU/rx
4% formalin 8 week	103±4	-3.25±0.09	43.3±0.3	>0,99	10 ⁶ –10 ¹ GU/rx	10 ² GU/rx

The values of Efficiency, Slope and Intercept are expressed as mean ± standard deviation calculated from two spiking experiments with *V. cholerae* ATCC 39315 cells, each quantified in triplicate on the same run (n = 6)

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However, nucleic acids isolated from formalin-fixed samples are most often degraded and contain small DNA fragments, generally less than 300 bp [45, 46]. Such small fragments make DNA often inaccessible to the PCR reaction [47, 48]. The fact that the developed primers amplify a short fragment of 206 bp makes them suitable also for the analysis of formalin-fixed samples.

The performance of the method was evaluated by applying the qPCR on genomic DNA extracted for a dilution series of 4% formalin-fixed *V. cholerae* ATCC 39315 spiked suspensions (10⁶–10¹ GU/rx) using the protocol we previously described for molecular analysis of historical CPR samples [25]. This protocol included specific steps such as incubation at an elevated temperature after proteinase K digestion that partially removes formalin crosslinking (which is also a result of formalin-fixation, leading to decreases in the accessibility of extracted DNA for enzymes such as DNA polymerase during PCR) thus allowing genomic DNA to be efficiently purified for subsequent molecular analyses [25]. As shown in Table 3, the qPCR was able to detect and quantify target DNA down to a level of 10¹ GU/rx (50 gene copies per reaction). The linearity of the performed assays was good (R² > 0.99) over a 6-log-unit dynamic range and the PCR amplification efficiencies ranged from 0.94 to 1.04.

The qPCR was also performed on serial dilutions (10⁶–10¹ GU/rx) of artificially fragmented *V. cholerae* ATCC 39315 genomic DNA samples composed of a homogenous pool of fragments having an average size of 276 bp (Fig 2A). This level of fragmentation was specifically

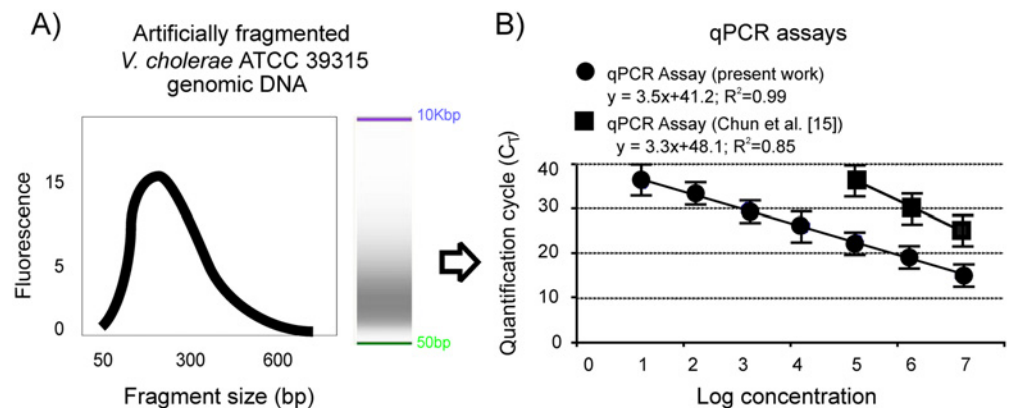


Fig 2. Performance of the qPCR assay for detection of *V. cholerae* in artificially degraded DNA samples. (A) Electropherogram plot obtained by Agilent Bioanalyzer analysis of artificially fragmented genomic DNA of *V. cholerae* ATCC 39315. (B) Plot of mean Cq-values from three replicates tested against the *V. cholerae* artificially fragmented DNA inputs. Error bars indicate the standard deviations of the means.

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selected in order to mimic DNA degraded conditions commonly encountered in DNA retrieved from historical formalin-fixed samples [45, 46] including CPR samples [25]. As shown in Fig 2B, qPCR performance on fragmented DNA was very good in terms of efficiency (0.91), linearity ($R^2 > 0.99$) and limit of detection (10^1 GU/rx) and comparable to the performance of the method with pure-culture DNA. In addition, the performance of the method with fragmented samples was much better than performance by one of the most commonly employed qPCR protocol applied on the same samples [15] (Fig 2B).

A case study: *V. cholerae* detection in Continuous Plankton Recorder (CPR) samples

The performance of the method was ultimately evaluated on historical formalin-fixed samples collected by the CPR survey during the last 60 years in several worldwide locations. The CPR Survey is one of the longest running marine biological monitoring programs in the world and provides a long-term archive of formalin-preserved plankton samples (<http://www.sahfos.ac.uk>). Because the plankton is one of the largest reservoirs of vibrios in nature, the molecular analysis of CPR samples has recently been proposed as a breakthrough approach to study the long-term ecology (over large temporal scales) and macro-ecology (over large spatial scales) of vibrios in the aquatic environment [49].

To test for the presence of PCR inhibitors the LightMix Modular PhHV spiked Extraction Control (Roche Diagnostics, Milan, Italy) was applied on DNA recovered from representative CPR samples (all samples scored negative for the qPCR test) collected at different times and geographic locations: 11IN2-2 (Irish Sea, 1971), 157SB-38 (Iberian Coast, 1971), 4CT4 (South Africa, 2011), 8CT30 (South Africa, 2011). A 85 bp long fragment from the Phocine herpesvirus (PhHV) sequence target was amplified with specific primers and detected with a LC670 labeled hydrolysis probe. Results from the test showed that no inhibitors were present in the tested DNA.

qPCR was firstly applied on two samples (413R and 485R) collected in the North Sea off the river Rhine estuary (51.9–52.41N; 3.3–4.01E) in 1998 and 2004, respectively. These samples were analyzed in a previous study by 16SrDNA amplicon pyrosequencing that revealed the presence of read sequences showing >95% identity to *V. cholerae* [25]. According to these results both 413R and 485R samples tested positive for qPCR. The method was then applied on 5 additional CPR samples that were collected by the Survey between 1961 and 1971 at different coastal locations of the North Sea and North Atlantic Ocean (S2 Table). Sample 157SB from the Bay of Biscay (47.5–48.5N; 4.5–5.5 W) in 1971 and sample 228A collected in the vicinity of the Shetland Islands (58–59N; 1–2 W) in 1966 scored positive to the test (S2 Table). Finally, the qPCR was run on 18 CPR samples collected in 2011 along the coasts of Angola, Namibia and South Africa, which represent endemic areas for cholera (Fig 3). Interestingly, sample 8CT28 (C_q = 32) collected in coastal water off the city of Port Elizabeth (South Africa), sample 7CTend (C_q = 33) collected off the city of Cape Town (South Africa) and sample 4CT6 (C_q = 33) collected off the city of Luanda (Angola) were positive to the test. All positive results (samples 413R-, 485R-, 228A-8, 157SB-2, 8CT28, 7CTend, 4CT6) were further confirmed through purification and sequencing of the amplified fragments showing that amplicon sequences fully matched (100% coverage and 100% identity) the *gbpA* gene sequence of *Vibrio cholerae* reference strain N16961 (GenBank accession number: EU072441.1).

Collectively, these results demonstrate the success of the developed assay in detecting *V. cholerae* DNA in historical formalin-fixed samples, the earliest of which dates back to August 1966. This finding may have important implications for the study of long-term ecology of *V. cholerae* in the aquatic environment including investigations aiming to shed light on the

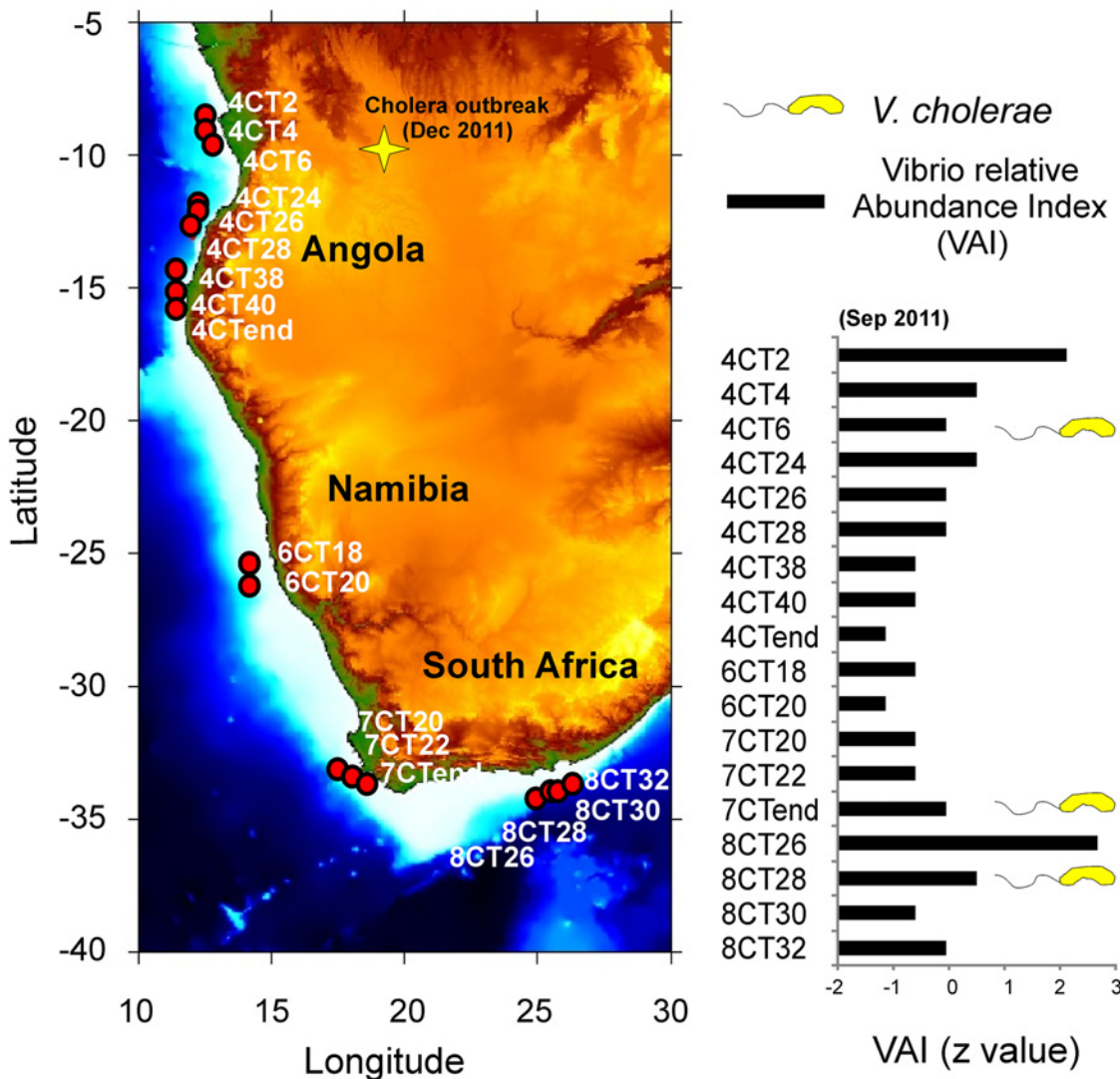


Fig 3. *V. cholerae* detection in CPR samples from endemic Cholera regions (South West Africa). Relative abundance of *Vibrio* spp. and *Vibrio cholerae* in CPR samples collected in cholera endemic areas of the Benguela Current Large Marine Ecosystem region (BCLME, South West Africa) as a proof of concept for the possible use of the CPR technology and the developed qPCR assay in cholera studies (see main text for details).

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effect of climate and environmental changes on the worldwide spreading of this bacterium and its associated diseases [49].

Broadly, the protocol may also be useful for the preliminary screening of *V. cholerae* O1, O139, Non-O1, and Non-O139 in formalin-fixed samples of particular ecological, evolutionary and/or epidemiological significance prior to being further analysed by the latest genotyping techniques such as whole genome targeted capture-enrichment methods coupled to next generation sequencing (NGS) approaches [50]. In this context, the detection of *V. cholerae* in three out of eighteen CPR samples collected in cholera endemic areas (e.g. sample 8CT28, 7CTend and 4CT 6), is of particular significance and represents a proof of concept for the possible use of the CPR technology in cholera studies (Fig 3).

Although “absolute quantification” of bacterial cells could not be attempted on CPR samples (due to the loss/damage of DNA that could not be taken into account), “relative quantification” such as the use of the VAI index may be applied (Fig 3). Such an index, which was

initially developed for the relative quantification of total vibrios in CPR samples [25], could be extended to measure and compare *V. cholerae* relative abundances in different geographic areas and time periods. For instance, CPR sampling and analysis of plankton and *V. cholerae* might be employed to study, for the first time, the macro-ecology of cholera, i.e. the relationships between the bacterium and its environment at a geographically extensive scale. Our understanding of such relationships has been constrained in the past mainly because sampling of *V. cholerae* in the environment was only obtained at single point sites and times, which do not capture the ecological background of the disease.

The use of the CPR in tracking cholera outbreaks and epidemics is also of great potential interest if we consider that one CPR sample alone (representing a tow over 10 nautical miles) is equivalent to multiple point samples typically employed in environmental cholera studies, thus providing orders of magnitude improvement in sampling coverage. Against this background, it is worth mentioning that more than 100 cases of cholera were registered during the first fortnight of December 2011 (2 months after the collection of CPR samples analyzed in this study) in the district of Lucapa (northern Angola) located 800 km away from Luanda (Fig 3). Genotyping of *V. cholerae* DNA recovered from this sample is currently in progress in our laboratory, although, at this stage, no direct or indirect connection can obviously be inferred.

Cholera research by the Continuous Plankton Recorder Survey is a collaborative development between the University of Genoa and the Sir Alister Hardy Foundation for Ocean Science (SAHFOS) and is expected to open up new research avenues addressing some important questions such as the role of human versus environmental factors in the origin, transmission and spreading of the cholera disease.

Conclusions

Overall the qPCR protocol developed in this work provides a new tool for the robust and sensitive detection and quantification of *V. cholerae* in problematic matrices such as environmental and stool samples. This has potential application for studies investigating the ecology and epidemiology of *V. cholerae* and in the field of public health. In addition, the method performs very well on formalin-fixed biological samples and is of great value for a wide range of applications including genetic, evolutionary, biogeographic, ecological and epidemiological retrospective studies. As a case study, the detection of *V. cholerae* in CPR samples collected in cholera endemic areas such as the Benguela Current Large Marine Ecosystem (BCLME) is of particular significance and represents a proof of concept for the possible use of the CPR technology and the developed qPCR assay in cholera studies.

Supporting Information

S1 Table. Species and strains tested.

(PDF)

S2 Table. qPCR detection of *Vibrio cholerae* in Continuous Plankton Recorder samples.

(PDF)

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Author Contributions

Conceived and designed the experiments: LV MS CP. Performed the experiments: LV MS CG EP. Analyzed the data: LV MS CG EP CP HMV NJPO. Contributed reagents/materials/analysis tools: LV CP HMV NJPO. Wrote the paper: LV MS.

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