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BIOTECHNOLOGY METHODS

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Molecular approach for the rapid detection of *Bacillus* and *Pseudomonas* genera—dominant antagonistic groups—from diverse ecological niches using colony multiplex PCR

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Abstract *Bacillus* and *Pseudomonas* are the dominant groups of bacteria known for their antagonistic potential against many plant and animal pathogens. Presently, exploration of these genera with antagonistic property for disease management of aquaculture system is gaining more importance to overcome the use of antibiotics and related resistance issues. Rapid screening and identification of these genera from diverse bacterial populations by conventional methods is laborious, cost-intensive, and time-consuming. To overcome these limiting factors, in the present study, a colony multiplex PCR (*cm*PCR) method was developed and evaluated for the rapid detection of *Bacillus* and *Pseudomonas*. The technique amplifies the partial 16S rRNA gene of *Bacillus* and *Pseudomonas* with a product size of ~1,100 and ~375 bp, respectively, using single forward (BSF2) and two reverse primers (PAGSR and BK1R). Reliability of the *cm*PCR method was confirmed by screening 472 isolates obtained from ten different eco-stations, of which 133 isolates belonged to *Bacillus* and 32 to *Pseudomonas*. The *cm*PCR method also helped to identify six different *Pseudomonas* spp. and 14 different *Bacillus* spp. from environmental samples. Of the total 472 isolates studied, 46 showed antagonistic activity, among which 63 % were *Bacillus* and 17.4 % were *Pseudomonas*. Thus, the newly developed molecular approach provides a quick, sensitive, and potential screening tool to detect novel, antagonistically important *Bacillus* and *Pseudomonas* genera for their use in aquaculture. Further, it can also act as a taxonomic tool to understand the distribution of these genera from wide ecological niches and their exploitation for diverse biotechnological applications.

Keywords *Bacillus* · Bacterial identification · Colony multiplex PCR · *Pseudomonas* · Taxonomical tool

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Introduction

Aquaculture is the fastest-growing food production sector in the world and accounts for approximately 47 % of the world's total fish food supply [9]. Disease outbreaks due to pathogenic bacteria have presented a major challenge, which adversely affects the development of aquaculture-based fish production [12]. The therapeutic options available in the control of bacterial diseases are the use of approved antibiotics, chemicals, and vaccination, but these applications are limited due to the development of antibiotic resistance and lack of consistency. Nowadays, the use of antagonistic bacteria such as *Bacillus*, *Pseudomonas*, *Alteromonas*, and *Flavobacterium* are also gaining importance in aquaculture industry to control bacterial diseases because of its ease in application and absence of side effects [3, 35, 40]. Our previous studies have clearly emphasized the importance of the genera *Bacillus* and *Pseudomonas*, with antagonistic activity and its application in aquaculture systems [22, 34]. These genera were also reported for its potential antimicrobial activity against many plant and human pathogens [23, 29].

Generally, the identification of bacteria by conventional methods is time-consuming, invariably mono-specific, as well as laborious, especially when screening a large number of field samples [6]. The methods like fatty acid methyl ester (FAME) profiling [4, 33] and the API system-based identification have been shown to be more useful than

classical methods [20]. However, these phenotypical protocols are again laborious, time-consuming, and are ineffective in rapid identification, with many non-specific results [38]. The disadvantages associated with these techniques could be overcome by employing alternative DNA-based detection methods that are generally faster, specific, and more reliable in identification [8, 24].

The 16S rDNA-based identification of bacteria has been widely recognized in studying distinct phylogenetic relationships and is effective in developing numerous taxonomical tools [21, 31]. Uniplex PCR-based assays have also been reported to be useful in the identification of bacteria [11, 30]; however, the rapid detection of multiple bacteria of interest in a single reaction is complicated, as it requires simultaneous amplification of more than one locus, and realizing the required specificity [21]. Compared to individual PCR assays, multiplex PCR allows the amplification and detection of multi-targeted genes in a single reaction, which minimizes the time, cost, and effort required for identification of different groups of bacteria, especially when studying a large number of field samples [1, 5, 25, 26].

Direct colony PCR is a useful approach that can avoid the difficulties encountered during the isolation of DNA. This method was found to be a quick, precise, and cost-effective tool for characterizing a large number of isolates from environmental samples [16]. Colony PCR was initially applied in bacteria [14] followed by yeast [37] and fungi [32], and eventually applied for detection purposes in higher organisms [36]. Earlier reports about the genus-specific primers for *Bacillus* [19, 39] and *Pseudomonas* [11, 30], and the 16S rDNA information of these genera obtained from the NCBI database, served as a base for developing a multiplex PCR assay for the simultaneous detection of both the genera. The present work was intended to develop a multiplex PCR assay in combination with colony PCR as a molecular screening tool for simultaneous identification of the genera *Bacillus* and *Pseudomonas* in a single reaction, and also to validate the reliability of the method to detect these genera from various ecological niches.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study belonged to the Microbial Culture Collection, Marine Biotechnology Division (MCC-MBTD), Central Marine Fisheries Research Institute (CMFRI), Cochin, India (Table 1). Apart from the above, 21 reference strains used in this study were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India, and Central Institute of Brackish water Aquaculture (CIBA), Chennai, India (Table 2). Bacterial strains were preserved in nutrient broth as glycerol stocks under −80 °C for further use.

Oligonucleotide design/primer selection

Generic level variations existing in 16S rDNA sequences were utilized for designing specific primers. Genus specific oligonucleotides were designed for *Bacillus* and *Pseudomonas* from the 16S rDNA sequences (72 *Bacillus* spp. and 92 *Pseudomonas* spp.) available in the GenBank database, including the submissions from our own collections. Multiple alignments of 16S rDNA sequences of *Bacillus*, *Pseudomonas* and its phylogenetically related strains were carried out with ClustalW using BioEdit software (version 7.1.3.0) [13]. The conserved sites from the aligned sequences were identified and the target specific primers were designed manually with an average length of 18–22 bp having an annealing temperature range between 55 and 62 °C. The performance of the designed primers was validated with the published primers as combinations. The oligonucleotide primers used were synthesized commercially (Sigma, India), and were suspended in sterile milliQ water and stored at −20 °C until use (Table 3).

DNA extraction

Multiplex PCR optimization was initially carried out with purified bacterial DNA. Bacterial cells (5 ml) grown (18–24 h old) were pelletized by centrifugation at 8,000 rpm for 10 min. The cells were re-suspended in 450 µl TEG (25 mM TrisHCl; 10 mM EDTA; 50 mM glucose) buffer (pH 8) containing lysozyme (5 mg/ml). The suspension was vortexed thoroughly and mixed with 35 µl of 10 % SDS. The tubes were then incubated on ice for 10 min, followed by the addition of 5 µl of proteinase K (20 mg/ml). This was further incubated at 60 °C in a water bath for 60 min. After the completion of cell lysis, the DNA was purified with the standard phenol–chloroform extraction method [28]. The isolated DNA was quantified using the Biophotometer (Eppendorf, Germany). Purified bacterial DNA was dissolved in 30 µl of TE buffer and stored at −20 °C for future use.

Uniplex PCR

All the 20 primers were individually tested and optimized for the conditions to amplify the genus *Bacillus* and *Pseudomonas*. The total reaction volume (25 µl) comprised of bacterial DNA (50 ng), 10 pmol each of two oligonucleotide primers, 2.5 mM of each deoxynucleoside triphosphate (Finnzymes), 1.5 U of Taq polymerase (Sigma), and 2.5 µl

Table 1 List of various strains used in this study and their identification details by various methods

Species	Number of isolates (*n*)	Source	Identification		
			Biochemical	16S rDNA sequencing	*cm*PCR assay
Pseudomonas aeruginosa	9	Sediment/water	*Pseudomonas* sp.	*Pseudomonas aeruginosa*	*Pseudomonas* sp.[a]
Pseudomonas fluorescens	2	Sediment	*Pseudomonas* sp.	*Pseudomonas fluorescens*	*Pseudomonas* sp.[a]
Pseudomonas mendocina	2	Fish/microalgae	*Pseudomonas* sp.	*Pseudomonas mendocina*	*Pseudomonas* sp.[a]
Pseudomonas pseudoalcaligenes	1	Sediment	*Pseudomonas* sp.	*Pseudomonas pseudoalcaligenes*	*Pseudomonas* sp.
Pseudomonas putida	5	Sediment/water	*Pseudomonas* sp.	*Pseudomonas putida*	*Pseudomonas* sp.[a]
Pseudomonas syringae	1	Water	*Pseudomonas syringae*	*Pseudomonas syringae*	*Pseudomonas* sp.
Pseudomonas sp.	5	Sediment	*Pseudomonas* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.
Bacillus amyloliquefaciens	14	Sediment/water	*Bacillus amyloliquefaciens*	*Bacillus amyloliquefaciens*	*Bacillus* sp.[a]
Bacillus aquimaris	3	Sediment	*Bacillus aquimaris*	*Bacillus aquimaris*	*Bacillus* sp.
Bacillus cereus	6	Fish/sediment	*Bacillus* sp.	*Bacillus cereus*	*Bacillus* sp.[a]
Bacillus cibi	1	Sediment	*Bacillus cibi*	*Bacillus cibi*	*Bacillus* sp.
Bacillus firmus	1	Sediment	*Bacillus* sp.	*Bacillus firmus*	*Bacillus* sp.[a]
Bacillus flexus	2	Fish/water	*Bacillus* sp.	*Bacillus flexus*	*Bacillus* sp.
Bacillus fusiformis	1	Fish	*Bacillus* sp.	*Bacillus fusiformis*	*Bacillus* sp.
Bacillus horikoshii	1	Sediment	*Bacillus* sp.	*Bacillus horikoshii*	*Bacillus* sp.
Bacillus licheniformis	2	Fish	*Bacillus licheniformis*	*Bacillus licheniformis*	*Bacillus* sp.[a]
Bacillus marisflavi	6	Sediment	*Bacillus marisflavi*	*Bacillus marisflavi*	*Bacillus* sp.
Bacillus megaterium	3	Fish/sediment	*Bacillus* sp.	*Bacillus megaterium*	*Bacillus* sp.
Bacillus pumilus	8	Sediment/microalgae	*Bacillus pumilus*	*Bacillus pumilus*	*Bacillus* sp.[a]
Bacillus sphaericus	1	Sediment	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus* sp.
Bacillus subtilis	24	Sediment/water	*Bacillus subtilis*	*Bacillus subtilis*	*Bacillus* sp.[a]
Bacillus sp.	2	Sediment	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.
Acinetobacter baumannii	1	Water	*Acinetobacter* sp.	*Acinetobacter baumannii*	No amplification
Acinetobacter sp.	2	Sediment	*Acinetobacter* sp.	*Acinetobacter* sp.	No amplification
Aeromonas veronii	1	Fish	*Aeromonas veronii*	*Aeromonas veronii*	No amplification
Aeromonas sp.	1	Sediment	*Aeromonas* sp.	*Aeromonas aquariorum*	No amplification
Alcaligenes faecalis	8	Sediment	*Alcaligenes* sp.	*Alcaligenes faecalis*	No amplification
Alcanivorax sp.	2	Water	*Alcanivorax* sp.	*Alcanivorax dieselolei*	No amplification
Arthrospira maxima	3	Water	*Arthrospira maxima*	*Arthrospira maxima*	No amplification
Brevibacterium sp.	2	Fish	*Brevibacterium* sp.	*Brevibacterium* sp.	No amplification
Citrobacter freundii	3	Fish	*Citrobacter* sp.	*Citrobacter freundii*	No amplification
Enterobacter sp.	3	Fish	*Enterobacter* sp.	*Enterobacter* sp.	No amplification
Enterococcus faecium	1	Fish	*Enterococcus* sp.	*Enterococcus faecium*	No amplification
Escherichia coli	2	Water	*Escherichia* sp.	*Escherichia coli*	No amplification
Halomonas sp.	1	Fish	*Halomonas* sp.	*Halomonas aquamarina*	No amplification
Micrococcus sp.	1	Water	*Micrococcus* sp.	*Micrococcus* sp.	No amplification
Nocardiopsis sp.	1	Sediment	*Nocardiopsis* sp.	*Nocardiopsis* sp.	No amplification
Oceanimonas sp.	1	Sediment	*Oceanimonas* sp.	*Oceanimonas doudoroffii*	No amplification
Pedobacter sp.	1	Sediment	*Pedobacter* sp.	*Pedobacter* sp.	No amplification
Pseudoalteromonas sp.	2	Fish	*Pseudoalteromonas* sp.	*Pseudoalteromonas* sp.	No amplification
Shewanella sp.	3	Oyster /water	*Shewanella* sp.	*Shewanella* sp.	No amplification
Streptomyces sp.	3	Sediment	*Streptomyces* sp.	*Streptomyces* sp.	No amplification
Vibrio alginolyticus	6	Water	*Vibrio* sp.	*Vibrio alginolyticus*	No amplification
Vibrio cholerae	2	Water	*Vibrio* sp.	*Vibrio cholerae*	No amplification

Table 1 continued

Species	Number of isolates (*n*)	Source	Identification		
			Biochemical	16S rDNA sequencing	*cm*PCR assay
Vibrio fluvialis	8	Sediment	*Vibrio fluvialis*	*Vibrio fluvialis*	No amplification
Vibrio sp.	2	Water	*Vibrio* sp.	*Vibrio* sp.	No amplification

[a] An isolate from each of these species was cross-checked by sequencing the *cm*PCR product. The sequenced results match the results of 16S rDNA

Table 2 Reference strains used in the study

Species	Strain code	Source	Identification	
			Biochemical and 16S rDNA sequencing	Multiplex PCR assay
Pseudomonas aeruginosa	MTCC1688	Microbial type culture collection, Chandigarh, India	*Pseudomonas aeruginosa*	*Pseudomonas* sp.
P. fluorescens	MTCC103		*P. fluorescens*	*Pseudomonas* sp.
Bacillus subtilis	MTCC441		*Bacillus subtilis*	*Bacillus* sp.
B. amyloliquefaciens	MTCC1270		*B. amyloliquefaciens*	*Bacillus* sp.
B. cereus	MTCC430		*B. cereus*	*Bacillus* sp.
B. licheniformis	MTCC6824		*B. licheniformis*	*Bacillus* sp.
Aeromonas hydrophila	MTCC1739		*Aeromonas hydrophila*	No amplification
Vibrio vulnificus	MTCC1145, MTCC1146		*Vibrio vulnificus*	
V. alginolyticus	MTCC4439		*V. alginolyticus*	
V. parahaemolyticus	MTCC451		*V. parahaemolyticus*	
V. anguillarum	O1& A1	Central Institute of Brackish water Aquaculture, Chennai, India	*V. anguillarum*	No amplification
V. harveyi	101, 102, LB203, LB208, LB166, and LB209		*V. harveyi*	
V. alginolyticus	101 and 102		*V. alginolyticus*	

of 10× PCR buffer. Annealing temperature was standardized to a range of 48–62 °C. The amplification was carried out in a Veriti thermal cycler (Applied Biosystems, UK) with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 45 s, and a final extension of 72 °C for 10 min. In each PCR reaction, a set of negative bacterial control (*Aeromonas hydrophila* MTCC1739, *Alcaligenes faecalis* MBTDCMFRI Af01, *Enterobacter* sp. MBTDCMFRI Eb01, *Enterococcus* sp. MBTDCMFRI Ec01, *Escherichia coli* DH5alpha, *Shewanella* sp. MBTDCMFRI Sh02 and *Vibrio vulnificus* MTCC1145) and a negative reaction control (without template DNA) was also maintained. The amplified products were separated by electrophoresis in 1.5 % agarose gel and visualized under UV transilluminator by staining with ethidium bromide. The size (bp) of the amplified product was calculated by comparing it with a standard molecular weight DNA marker (Step-up 100 bp DNA ladder, Merck, India) using the software Image Lab version 3 (Bio-Rad, Hercules, CA, USA).

Multiplex PCR

Based on the specificity and product size obtained from uniplex reactions, two pairs of primers were selected for multiplex PCR optimization. Variables such as annealing temperatures (gradient from 48 to 62 °C, with an interval of 2 °C), $MgCl_2$ concentrations (1.5–3 mM) and primer concentrations were optimized with the ability to produce good intensity and specific bands with each targeted DNA. The reaction mixture composition and the amplification conditions are the same as in uniplex PCR except for the addition of $MgCl_2$, combination of multiple primers, and a change in annealing temperature. A negative reaction control (without template DNA) and a positive control (50 ng/μl) of purified DNA of reference strains (*B. subtilis* MTCC441 and *P. aeruginosa* MTCC1688) were also included in each batch of the PCR reaction. The amplified products were detected in 1.5 % agarose gel stained with ethidium bromide by gel electrophoresis.

Table 3 Genus-specific primers used in this study to identify the genera *Bacillus* and *Pseudomonas* spp.

Primers	Sequence (5′-3′)	Length (nt)	Product size (bp)	References
Target genus—*Pseudomonas*				
PsF	TTA GCT CCA CCT CGC GGC	18	960	Garbeva et al. [11]
PsR	GGT CTG AGA GGA TGA TCA GT	20		
PA-GS-F	GAC GGG TGA GTA ATG CCT A	19	618	Spilker et al. [30]
PA-GS-R	CAC TGG TGT TCC TTC CTA TA	20		
PSF1	GGT CTG AGA GGA TGA TCA G	19	Present study	
PSF2	ACA CTG GAA CTG AGA CAC GG	20		
PSF6	CGG AAT TAC TGG GCG TAA A	19		
PSR1	CGT GGA CTA CCA GGG TAT CTA	21		
PSR6	GCC GTA AGG GCC ATG ATG A	19		
PSR7	ATT ACT AGC GAT TCC GAC TTC	21		
Target Genus—*Bacillus*				
Bac-F	CGG CGT GCC TAA TAC ATG CAA G	22	1,200	Kwon et al. [19]
Bac-R	GGC ATG CTG ATC CGC GAT TAC TA	23		
B-K1/F	TCA CCA AGG CRA CGA TGC G	19	1,114	Wu et al. [39]
B-K1/R	CGT ATT CAC CGC GGC ATG	18		
BSF1	ACA CTG GGA CTG AGA CAC G	19	Present study	
BSF2	TAC GGG AGG CAG CAG TRG G	19		
BSF6	GAG GAA CAC CAG TGG CGA A	19		
BSR1	CCA GGG TAT CTA ATC CTG T	19		
BSR2	CCG TCA ATT CCT TTG AGT TT	20		
BSR5	GTT GCG CTC GTT GCG GGA	18		

Colony multiplex PCR

This protocol is based on the crude DNA obtained from boiling water bath method described by Wan et al. [36]. Briefly, the single bacterial colony (18–24 h old) was picked with an autoclaved toothpick and mixed with 100 µl of TE buffer. The mixture was heated in a boiling water bath at 100 °C for 10 min. Cells were vortexed and centrifuged at 10,000 rpm for 2 min. One microliter of supernatant was used as template for a 10-µl PCR reaction mix. Thermal cycling conditions and reaction mixture preparation were similar to that of multiplex PCR conditions.

Sensitivity assay

Sensitivity of the multiplex PCR assay was tested for both purified and crude DNA obtained from bacterial suspensions. The ability to detect the lowest levels for multiplex PCR amplification (limits of detection) was performed in duplicates. Sensitivity of the multiplex PCR assay was evaluated using a series of targeted genomic DNA by decimally diluting the purified template DNA (concentration 50 ng) in sterile water. From the dilutions, 1 µl each was used as DNA template to carry out the multiplex (combinational primers) assay. Similarly, for detecting the sensitivity with the crude DNA, a single colony was picked from the cultures (18–24 h old) grown on nutrient agar and the bacterial suspensions were prepared by dissolving it in 1 ml of sterile saline (0.85 % NaCl) then serially diluted (up to 10^{-7} fold). These dilutions (1 µl per reaction volume) were used to determine the concentration (count) of each bacterium to get amplified in *cm*PCR assay. The concentration of each bacterium was determined by surface plating (100 µl) of appropriate dilutions into nutrient agar plates (Himedia, India). The plates were kept in incubation at 30 °C for 18–24 h. The counts were taken from each plate and the corresponding CFU/µl present in each dilution was calculated for the reaction.

Specificity assay

The specificity of colony multiplex PCR in identification of the genus *Bacillus* and *Pseudomonas* was examined with purified and crude DNA obtained from bacterial colonies (separately or in combination) representing different genera. The assay was carried out with the isolates of different genera obtained from the MCC-MBTD, CMFRI by keeping *Bacillus* and *Pseudomonas* as positive controls (Table 1).

Screening of environmental isolates

To evaluate the usefulness of the developed *cm*PCR assay, the protocol was applied to screen the bacteria

isolated from a wide range of environments. The sediment samples were collected from eight different stations (Chettuva, Puthuvype and Mangalavanam—mangrove-associated environments; Edavanakkad, Mulavukad, Njarakkal, and Andhakaranazhi—fish farming environments and Perandoor—polluted canal), water samples from two stations [oyster hatchery (Krishi Vigyan Kendra, Njarakkal) and ornamental fish hatchery (West coast, Andhakaranazhi)], and also from oyster (*Crassostrea madrasensis*) and fishes from farming environments were used in the study (Fig. 1). The sample processing and isolation protocols were carried out as described by Nair et al. [22]. Briefly, bacteria were isolated by serially diluting the samples and plated in Zobell's marine agar. After 24–48 h of incubation, the colonies with distinct morphology were selected and purified. The efficacy of the multiplex PCR protocol in the detection of environmental isolates were confirmed by performing simultaneous assay using both purified DNA and colony PCR method. The isolates that were identified using 16S rDNA were preserved as glycerol stocks under −80 °C.

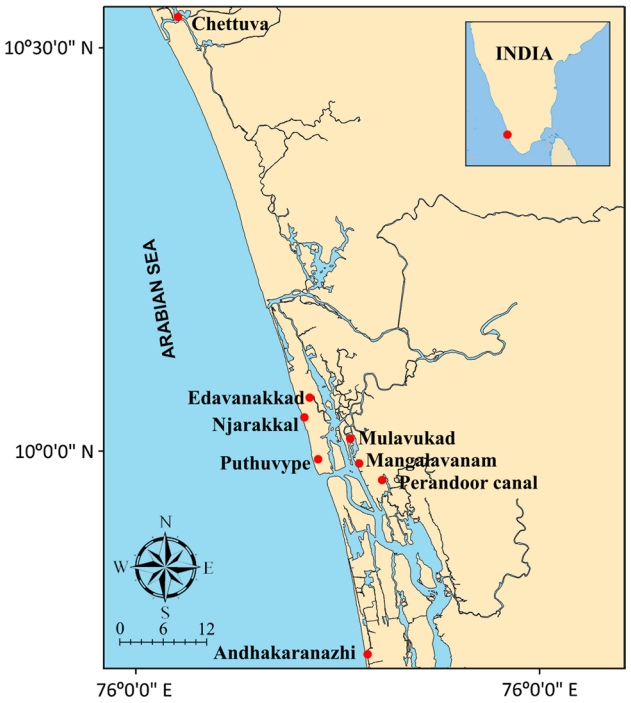

Fig. 1 Map showing the sample collection sites from the southwest coast of India

Detection of antagonistic activity

Antagonistic activity of the isolated bacteria was screened against aquaculture pathogens by using the spot diffusion method [22]. Briefly, targeted bacteria were spotted over pre-swabbed plates with aquaculture pathogens viz., *V. vulnificus* MTCC1145, *V. harveyi* 101, *V. anguillarum* O1, *V. parahaemolyticus* MTCC451, and *V. alginolyticus* 101. After incubation, the bacteria having notable antagonistic potential (zone of clearance of 10 mm or greater observed around isolates) were selected, identified, and preserved for further use.

Identification of bacteria

The consistency and specificity of *cm*PCR was verified by selecting 160 isolates at random (amplified and non-amplified strains in multiplex PCR assay) for identification using standard biochemical [17] and molecular (16S rDNA) [22] methods. The amplified products were purified (HiPurA PCR product purification kit, Himedia) and sequenced. Sequences of 16S rDNA fragments were imported to BLASTn [2] for similarity searching with available sequences in the GenBank database at NCBI. The sequences obtained were submitted in the GenBank for accession. Similarly, the products obtained through *cm*PCR were also sequenced and the results were cross checked.

Results

Uniplex PCR optimization

A total of ten primer sets was selected for this study, of which six primer sets were designed using the information already available in the GenBank and four primer pairs were reported elsewhere. Among these, five primer sets were intended to amplify *Pseudomonas* spp. and rest for *Bacillus* spp. (Table 3). Uniplex PCR optimization employing each primer pair produced amplified products ranging from 115 to 1,200 bp in size. The primer pairs, which yielded amplicons sufficient to differentiate the genera *Bacillus* and *Pseudomonas*, were picked for optimizing multiplex PCR. Among the 20 primers used, four pairs of primers (PSF2 and PSR1, PAGSF and PAGSR, BSF2 and BSR5, and BK1F and BK1R) were chosen for multiplex PCR with an optimal annealing temperature at 59 °C (Figs. 2, 3).

Multiplex PCR optimization

Through multiplex PCR, successful amplification of the targeted region with highest sensitivity was obtained at annealing temperature 58 °C with 3 mM $MgCl_2$

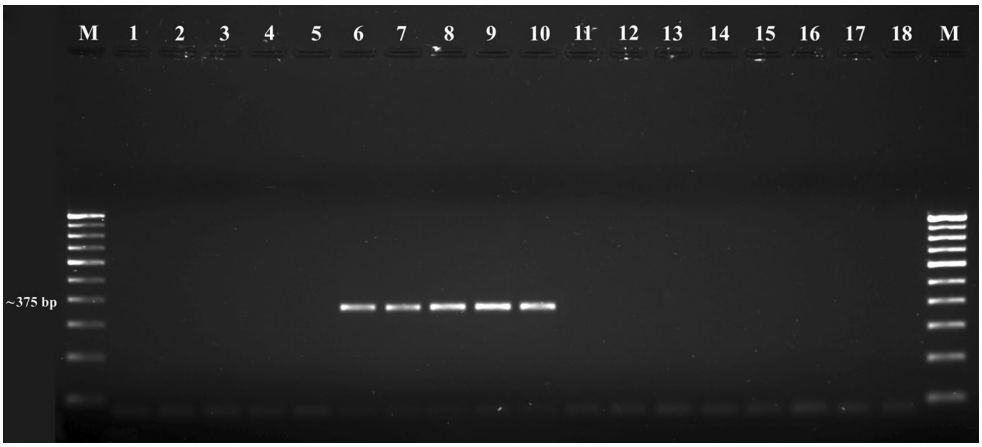

Fig. 2 The specificity of the uniplex PCR assay developed for the detection of *Pseudomonas* spp. using PSF2 and PAGSR. *Lane M*: molecular size marker (100-bp ladder); *lane 1*: *Bacillus subtilis* MTCC441; *lane 2*: *Bacillus amyloliquefaciens* MTCC1270; *lane 3*: *Bacillus licheniformis* MTCC6824; *lane 4*: *Bacillus cereus* MTCC430; *lane 5*: *Bacillus pumilus* MBTDCMFR1 Ba01; *lane 6*: *Pseudomonas aeruginosa* MTCC1688; *lane 7*: *Pseudomonas fluorescens* MTCC103; *lane 8*: *Pseudomonas putida* MBTDCMFRI Ps20; *lane 9*: *Pseudomonas mendocina* MBTDCMFRI Ps21; *lane 10*: *Pseudomonas* sp. MBTDCMFRI Ps18; *lane 11*: *Aeromonas hydrophila* MTCC1739; *lane 12*: *Vibrio vulnificus* MTCC1145; *lane 13*: *Enterobacter* sp. MBTDCMFRI Eb01; *lane 14*: *Escherichia coli* DH5alpha; *lane 15*: *Enterococcus* sp. MBTDCMFRI Ec01; *lane 16*: *Alcaligenes faecalis* MBTDCMFRI Af01; *lane 17*: *Shewanella* sp. MBTDCMFRI Sh02; *lane 18*: negative control of the reaction (*without template*)

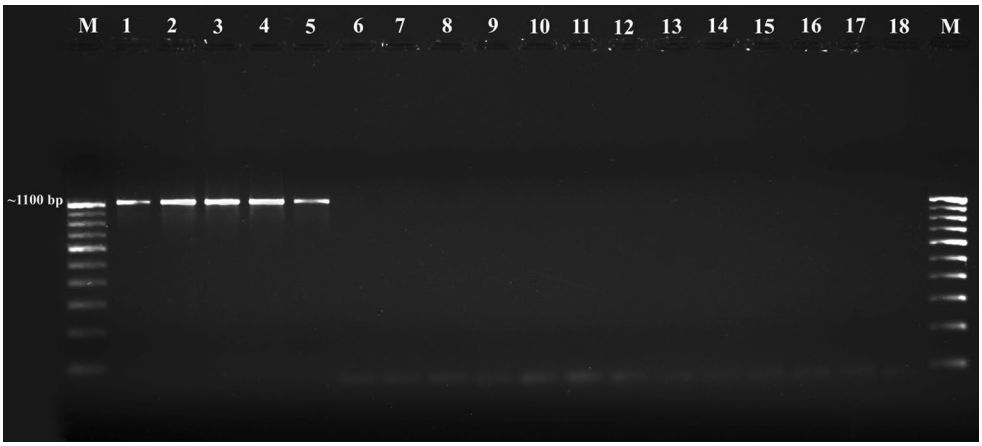

Fig. 3 The specificity of the uniplex PCR assay developed for the detection of *Bacillus* spp. using BSF2 and BKIR. *Lane M*: molecular size marker (100 bp ladder); *lane 1*: *Bacillus subtilis* MTCC441; *lane 2*: *Bacillus amyloliquefaciens* MTCC1270; *lane 3*: *Bacillus licheniformis* MTCC6824; *lane 4*: *Bacillus cereus* MTCC430; *lane 5*: *Bacillus pumilus* MBTDCMFR1 Ba01; *lane 6*: *Pseudomonas aeruginosa* MTCC1688; *lane 7*: *Pseudomonas fluorescens* MTCC103; *lane 8*: *Pseudomonas putida* MBTDCMFRI Ps20; *lane 9*: *Pseudomonas mendocina* MBTDCMFRI Ps21; *lane 10*: *Pseudomonas* sp. MBTDCMFRI Ps18; *lane 11*: *Aeromonas hydrophila* MTCC1739; *lane 12*: *Vibrio vulnificus* MTCC1145; *lane 13*: *Enterobacter* sp. MBTDCMFRI Eb01; *lane 14*: *Escherichia coli* DH5alpha; *lane 15*: *Enterococcus* sp. MBTDCMFRI Ec01; *lane 16*: *Alcaligenes faecalis* MBTDCMFRI Af01; *lane 17*: *Shewanella* sp. MBTDCMFRI Sh02; *lane 18*: negative control of the reaction (*without template*)

concentrations. Among the selected primers, the primers PSF2, PAGSR, BSF2, and BK1R were chosen for optimization of multiplex reactions. The rest of the primers (PSR1, PAGSF, BSR5, and BK1F) were omitted due to the occurrence of non-specific bands and cross-amplification with other genera during multiplex assays. The details of primer-binding regions in the genera *Bacillus* and *Pseudomonas* are shown in Fig. 4. The non-specific band obtained from the mixture of selected two primer pairs was limited by removing single forward primer (PSF2). The precise amplification for multiplex PCR was attained with the use of a single forward (BSF2) and two reverse (BK1R and PAGSR) primers at a concentration of 0.5 pmol/μl. These primer combinations yielded a distinguishable

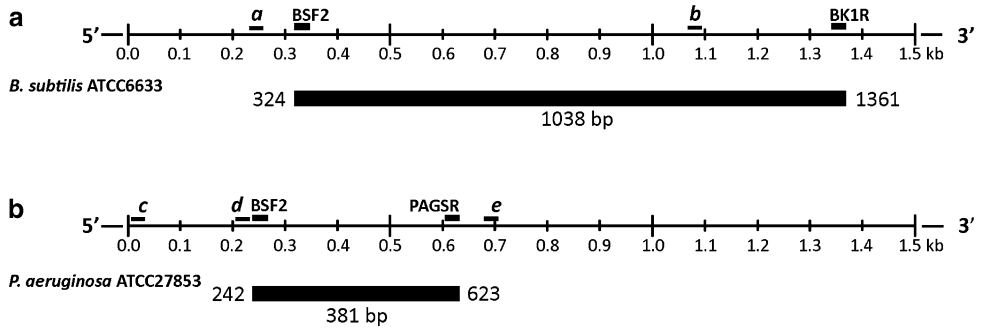

Fig. 4 Map represents the relative binding position of the primers in 16S rDNA sequence of *Bacillus* and *Pseudomonas*. Primers: *a* BK1F, *b* BSR5, *c* PAGSF, *d* PSF2, and *e* PSR1 The *lower bar* represents the amplified product of *Bacillus subtilis* ATCC6633 (**a**) and *Pseudomonas aeruginosa* ATCC27853 (**b**) by using multiplex PCR primers

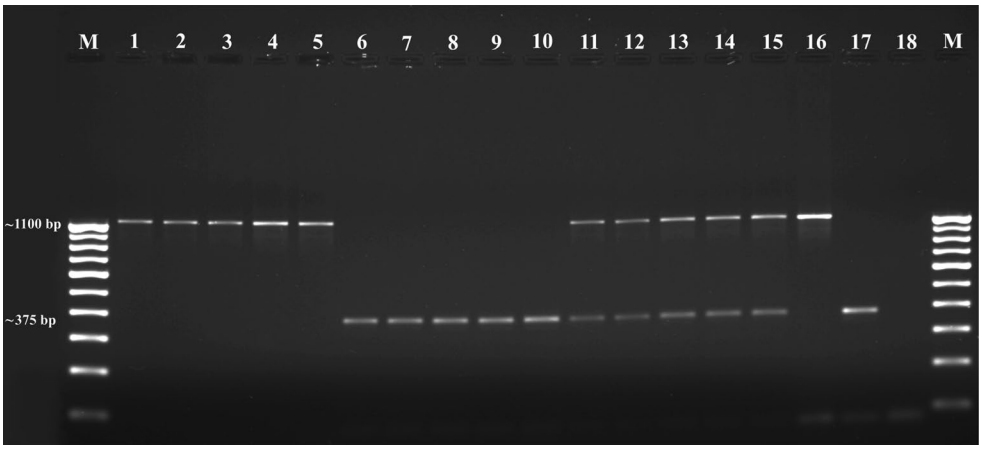

Fig. 5 The specificity of the mPCR assay developed for the detection of the genera *Bacillus* and *Pseudomonas* by using unitemplate and multitemplate DNA. PCR product size obtained for *Bacillus* spp. and *Pseudomonas* spp. are ~1,100 and ~375 bp, respectively. *Lane M*: molecular size marker (100 bp ladder); *lane 1*: *Bacillus subtilis* MTCC441; *lane 2*: *Bacillus amyloliquefaciens* MTCC1270; *lane 3*: *Bacillus licheniformis* MTCC6824; *lane 4*: *Bacillus cereus* MTCC430; *lane 5*: *Bacillus pumilus* MBTDCMFR1 Ba01; *lane 6*: *Pseudomonas aeruginosa* MTCC1688; *lane 7*: *Pseudomonas fluorescens* MTCC103; *lane 8*: *Pseudomonas putida* MBTDCMFRI Ps20; *lane 9*: *Pseudomonas mendocina* MBTDCMFRI Ps21; *lane 10*: *Pseudomonas* sp. MBTDCMFRI Ps18; *lane 11*: *Pseudomonas aeruginosa* MTCC1688 and *Bacillus subtilis* MTCC441; *lane 12*: *Pseudomonas putida* MBTDCMFRI Ps20 and *Bacillus amyloliquefaciens* MTCC1270; *lane 13*: *Pseudomonas fluorescens* MTCC103 and *Bacillus cereus* MTCC430; *lane 14*: *Pseudomonas mendocina* MBTDCMFRI Ps21 and *Bacillus licheniformis* MTCC6824; *lane 15*: *Pseudomonas* sp. MBTDCMFRI Ps18 and *Bacillus pumilus* MBTDCMFR1 Ba01; *lane 16*: *Bacillus subtilis* MTCC441 and *Vibrio vulnificus* MTCC1145; *lane 17*: *Pseudomonas aeruginosa* MTCC1688 and *Enterococcus* sp. MBTDCMFRI Ec01; *lane 18*: negative control of the reaction (*without template*)

product of ~1,100 and ~375 bp for *Bacillus* spp. and *Pseudomonas* spp., respectively (Fig. 5).

Multiplex PCR amplification using purified and crude DNA gave similar product quality under the same reaction conditions. By using the crude DNA extraction and the current mPCR method, the identification of the genera *Bacillus* and *Pseudomonas* from an unidentified bacterial culture can be completed within 2 h.

Sensitivity assay

The intensity of amplicon qualitatively decreased with the decrease in DNA concentration and also with CFU in dilutions (Fig. 6). The detection limit for *Bacillus* spp. and *Pseudomonas* spp. varied considerably in both purified DNA and crude DNA. The detection limits of the genomic DNA (purified and crude) in the multiplex PCR varied among different bacterial species and are listed in Table 4.

Specificity assay

The specificity was determined by using the *cm*PCR protocol on 239 bacterial isolates from MCC-MBTD, CMFRI (Table 1). The results were also confirmed with the reference strains mentioned in Table 2. None of the non-targeted genera produced any cross-reactive or non-specific

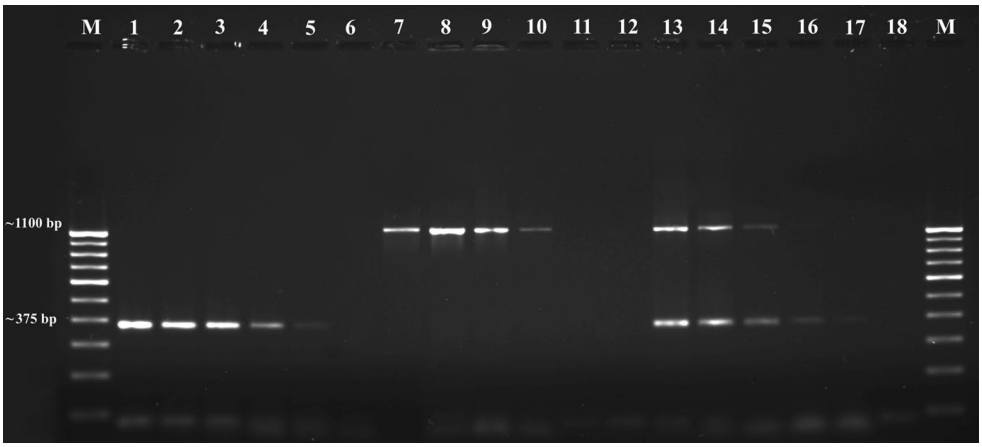

Fig. 6 The sensitivity of detection of *Bacillus* sp. and *Pseudomonas* sp. by mPCR assay. PCR amplification was carried out using tenfold-serially diluted template DNA of *Bacillus licheniformis* MTCC6824 and *Pseudomonas aeruginosa* MTCC1688. The size of the PCR products obtained were ~1,100 and ~375 bp for the genera *Bacillus* and *Pseudomonas*, respectively. *Lane M*: molecular size marker (100-bp ladder); *lane 1–6*: *Pseudomonas aeruginosa* MTCC1688 DNA serially diluted to 53 ng, 5.3 ng, 0.53 ng, 53 pg, 5.3 pg, and 0.53 pg; *lane 7–12*: *Bacillus licheniformis* MTCC6824 DNA serially diluted to 46 ng, 4.6 ng, 0.46 ng, 46 pg, 4.6 pg, and 0.46 pg, respectively; *lane 13–17*: serially diluted template of *Bacillus licheniformis* MTCC6824 and *Pseudomonas aeruginosa* MTCC1688; *lane 18*: negative control of the reaction (*without template*)

Table 4 Limit of detection for various species identified using multiplex PCR sensitivity assay

S. no.	Bacteria	Detection limit	
		pg/μl	CFU/μl
1.	*Bacillus amyloliquefaciens* MTCC1270	35	900
2.	*B. cereus* MTCC430	48	300
3.	*B. licheniformis* MTCC6824	46	580
4.	*B. pumilus* MBTDCMFRI Ba33	48	670
5.	*B. subtilis* MTCC441	51	1,000
6.	*Pseudomonas aeruginosa* MTCC1688	5.3	9
7.	*P. fluorescens* MTCC103	3.3	7
8.	*P. mendocina* MBTDCMFRI Ps21	4.2	13
9.	*P. putida* MBTDCMFRI Ps20	3.1	3
10.	*Pseudomonas* sp. MBTDCMFRI Ps18	8.8	11

results while carrying out the specificity assay, which in turn showed the higher specificity of the current *cm*PCR primers towards the genera *Bacillus* and *Pseudomonas*. The BLASTn results of the primer sequences selected also showed maximum homogeneity to the targeted genera when compared to others. ClustalW alignment of sequences of mPCR primers with the 16S rDNA sequence of *Bacillus*, *Pseudomonas*, and other genera also showed the accuracy of primer combination (Table 5).

Application of *cm*PCR for environmental samples

A total of 711 bacterial isolates were subjected to *cm*PCR assay, of which 234 were *Bacillus* spp. and 63 were *Pseudomonas* spp. The details of the isolates and their sources are given in Table 6. Among the isolates obtained from culture collection, 74 possessed antibacterial activity. Apart from this, 46 (9.7 %) of 472 environmental isolates also exhibited antagonistic activity towards aquaculture pathogens. The majority of them belonged to the genera *Bacillus* (63 %) followed by *Pseudomonas* (17.4 %) (Table 6).

To confirm the consistency of detection, 160 isolates (amplified and non-amplified strains) were selected based on their antagonistic activity and *cm*PCR results. The results of the selected isolates were cross checked with their biochemical and 16S rDNA data. Upon comparison of the results of identification (biochemical methods and 16S rDNA sequencing), the specificity and reliability of *cm*PCR in the detection of the genera *Bacillus* and *Pseudomonas* were found to be 100 % efficient. Among the 160 bacterial strains, 100 isolates that showed positive amplification in the *cm*PCR assay were *Bacillus* spp. (75) and *Pseudomonas* spp. (25). The rest of the 60 isolates that failed to amplify in *cm*PCR were identified as *Aeromonas*, *Vibrio*, *Alcaligenes*, *Enterobacter*, *Halomonas*, *Citrobacter*, *Enterococcus*, *Shewanella*, *Pseudoalteromonas*, *Escherichia*, *Arthrospira*, *Acinetobacter*, *Micrococcus*, *Alcanivorax*, *Pedobacter*, *Oceanimonas*, *Brevibacterium*, and *Streptomyces* (Table 1). Thus, the primer combinations developed for multiplex PCR assay were highly specific and sensitive by producing distinct bands for precise identification of the genera *Bacillus* and *Pseudomonas*.

While sequencing the *cm*PCR products of selected strains, the sequences exhibited similar identification

Table 5 Alignment of the primer sequences of BSF2, PAGSR, and BK1R with 16S rDNA sequences of 32 strains belonging to *Pseudomonas*, *Bacillus*, and other genera commonly found in the environment

Primer	BSF2		PAGSR		BK1R	
	10		10	20	10	
Sequence (5'-3')	TACGGGAGGC	AGCAGTRGG	TATAGGAAGG	AACACCAGTG	CATGCCGCGG	TGAATACG
Pseudomonas aeruginosa	. . . . .	. . . . .	. . . . .	. . . . .	A. - - T. A. . .	. . . . .
P. aeruginosa MBTDCMFRI Ps08	. . . . .	. . . . .	. . . . .	. . . . .	A. - - T. A. . .	. . . . .
Pseudomonas fluorescens	. . . . .	. . . . .	. . . . .	. . . . .	A. - - T. A. . .	. . . . .
Pseudomonas mendocina	. . . . .	. . . . .	. . . . .	. . . . .	A. - - T. A. . .	. . . . .
Pseudomonas putida	. . . . .	. . . . .	. . . . .	. . . . .	A. - - T.	. . . . .
Pseudomonas stutzeri	. . . . .	. . . . .	. . . . .	. . . . .	A. - - T.	. . . . .
Pseudomonas syringae	. . . . .	. . . . .	. . . . .	. . . . .	A. - - T.	. . . . .
Bacillus amyloliquefaciens	. . . . .	. . . . .	G. . GT. G. .	. . . . .	. . . . .	. . . . .
Bacillus cereus	. . . . .	. . . . .	G. . . T. G. .	. . . . .	. . . . .	. . . . .
Bacillus firmus	. . . . .	. . . . .	G. . GT. G. .	. . . . .	. . . . .	. . . . .
Bacillus licheniformis	. . . . .	. . . . .	G. . GT. G. .	. . . . .	. . . . .	. . . . .
Bacillus megaterium	. . . . .	. . . . .	G. . GT. G. .	. . . . .	. . . . .	. . . . .
Bacillus pumilus	. . . . .	. . . . .	G. . GT. G. .	. . . . .	. . . . .	. . . . .
Bacillus subtilis	. . . . .	. . . . .	G. . GT. G. .	. . . . .	. . . . .	. . . . .
B. subtilis MBTDCMFRI Ba37	. . . . .	. . . . .	G. . . T. G. .	. . . . .	. . . . .	. . . . .
Aeromonas hydrophila	. . . . .	. . . . .	G. . CT. G. .	. . T. . . G. .	A. . . TT. . .	. . . . .
Alteromonas genovensis	. . . . .	. . . . .	. . . CT. G. .	. . . . T. . A. .	A. . ATT. . .	. . . . .
Citrobacter freundii	. . . . .	. . . . .	G. . CT. G. .	. . T. . . N. .	A. . . . . A. .	. . . . .
Corynebacterium bovis	. . . . .	. . . . .	. . . CA. G. .	. . . . . GA. .	. . C. . T. . .	. . . . .
Enterobacter hormaechei	. . . . .	. . . . .	G. . CT. G. .	. . T. . . G. .	A. . . . . A. .	. . . . .
Enterococcus sp.	. . . . .	. . . . .	. . . . T. G. .	. . . . .	. . C.	. . . . .
Escherichia coli	. . . . .	. . . . .	G. . CT. G. .	. . T. . . G. .	A. . . . . A. .	. . . . .
Klebsiella oxytoca	. . . . .	. . . . .	G. . CT. G. .	. . T. . . G. .	A. . . . . A. .	. . . . .
Listeria monocytogenes	. . . . .	. . . . .	CG. . AAGC. C	GCGCAGGCG.	. . . . . A. .	. . . . .
Micrococcus luteus	. . . . .	. . . . .	. . . CA. G. .	. . . . . GA. .	. . C. . T. . .	. . . . .
Moraxella caprae	. . . . .	. . . . .	G. . CT. G. .	. . T. . . GA. .	A. - - T. . . .	. . . . .
Pseudoalteromonas sp.	. . . . .	. . . . .	G. . CT. . . .	. . T. . . GA. .	A. . . A. . . .	. . . . .
Serratia marcescens	. . . . .	. . . . .	G. . CT. G. .	. . T. . . G. .	A. . . . TA. .	. . . . .
Staphylococcus aureus	. . . . .	. . . . .	G. . . T. G. .	. . . . .	. . . . . TA. .	. . . . .
Streptococcus bovis	. . . . .	. . . . .	. . . . T. G. .	. . . . . G. .	. . C.	. . . . .
Vibrio harveyi	. . . . .	. . . . .	G. . CT. . . .	. . T. . . G. .	A. . . . . A. .	. . . . .
Yersinia pestis	. . . . .	. . . . .	G. . CT. G. .	. . T. . . G. .	A. . . . TA. .	. . . . .

results as 16S rDNA sequencing (Table 1). This suggests that the products of *cm*PCR can also utilize for identification of *Bacillus* and *Pseudomonas* up to species level by sequencing when required. From the present study, six different isolates of *Pseudomonas* spp. and 14 different isolates of *Bacillus* spp. have been identified precisely. The accession numbers of the sequences submitted in GenBank were JF719759 to JF719808 (49 submissions) and KF317775 to KF317832 (57 submissions).

Discussion

A specific and sensitive DNA-based identification method—*cm*PCR—for the rapid screening of antagonistically important genera *Bacillus* and *Pseudomonas* from environmental samples was developed in the present study. These two genera were reported to be ubiquitous and possess a wide spectrum of antagonistic activity towards various pathogens [3, 23, 29]. The significance of these genera in the control of bacterial diseases in aquaculture systems was clearly depicted in our previous studies [22, 34]. The development of a consortium of antagonistic bacteria and their characterization offers a better alternative approach in developing novel microbial products for the control of bacterial diseases and to tackle the issue of antibiotic resistances, which requires screening of *en masse* of bacterial populations from diverse ecosystems. In this background, the present report on a novel molecular method is of importance; to identify the isolates belonging to the promising genera of *Bacillus* and *Pseudomonas,* as an alternate to conventional diagnostic methods.

The current *cm*PCR assay employed the primer combinations targeting 16S rDNA region for simultaneous detection of *Bacillus* spp. and *Pseudomonas* spp. without noises from other genera. This region was widely accepted to infer phylogenetic relationships among bacteria and offers the benefit of robust databases and well-characterized phylogenetic primers [27]. Though 16S rDNA sequencing was widely used in bacterial taxonomy, its power to distinguish the species in certain genera was reported as poor [15]. The present molecular screening tool involves a combination of colony PCR and multiplex PCR to identify both the genera in a rapid, specific, sensitive, and cost-effective mode. It demonstrates the use of purified bacterial colony to perform multiplex PCR instead of isolated DNA to identify

Table 6 Results of the colony multiplex PCR (*cm*PCR) and the antagonistic activity of the isolates obtained from the southwest coast of India

Sampling site	Source	Sample	Number of isolates	Number of isolates detected positive in *cm*PCR		Number of antagonistic isolates detected by spot diffusion assay		
				Bacillus	*Pseudomonas*	*Bacillus*	*Pseudomonas*	Others
Andhakaranazhi	Ornamental fish hatchery	Water	37	0	1	0	0	0
		Fish	8	0	0	0	0	0
	Polluted canal	Sediment	36	18	1	4	0	0
Chettuva	Mangrove	Sediment	82	35	8	6	0	2
Edavanakkad	Canal	Sediment	20	1	2	0	1	0
Mandapam	Fish hatchery	Fish	14	5	1	0	0	0
Mangalavanam	Mangrove	Sediment	52	28	9	3	5	1
Mulavukad	Shrimp pond	Sediment	18	4	0	2	0	1
Njarakkal	Oyster hatchery	Water	21	0	0	0	0	1
		Oyster	44	1	6	0	0	0
Perandoor	Polluted canal	Sediment	37	12	0	4	0	1
Puthuvype	Mangrove	Sediment	22	4	0	2	0	1
	Canal	Sediment	17	5	0	1	0	0
	Refinery	Sediment	46	17	2	6	0	0
	Polluted canal	Water	18	3	2	1	2	2
			472	133	32	29	8	9
CMFRI	Microbial culture collection, MBTD	Culture collection	239	101	31	56	12	6
Total number of isolates screened			711	234	63	85	20	15

Bacillus spp. and *Pseudomonas* spp. This study also validated that the "boiling water bath method" is an appropriate method for the preparation of crude bacterial DNA to be used in PCR reactions, for both *Bacillus* spp. and *Pseudomonas* spp. The assay described for the identification is much simpler and the time required to complete the whole process (DNA preparation, amplification, and detection) was approximately 2 h. Our result is in agreement with the report of Kwon et al. [18] that the direct bacterial suspensions produce precise amplification and distinguishable PCR products.

For a successful multiplex PCR, a balance between primer combinations, magnesium chloride concentrations, and annealing temperatures are important. These factors differed even with uniplex and multiplex systems, which were also reported by Chen et al. [6]. The present study was successful in developing an efficient multiplex combinational primer mix using a single forward and two reverse primers providing high sensitivity and specificity in identifying the targeted genera *Bacillus* and *Pseudomonas*. In addition, the developed assay allowed the detection with <10 and 50 pg for genomic DNA and 1×10^1 and 7×10^2 CFU/μl for *Pseudomonas* spp. and *Bacillus* spp. respectively, which is comparable with results of Chiang et al. [7] and Fan et al. [10].

The reliability of bacterial identification by *cm*PCR assay was also checked and evaluated by comparing the results of 16S rDNA sequencing and biochemical methods. The results confirmed that the *cm*PCR assay developed in the present study is successful in the identification of the genera *Bacillus* and *Pseudomonas*. Moreover, the identification by sequencing the *cm*PCR product exactly matches with the results of 16S rDNA sequencing. Results observed with multiplex PCR and conventional methods were highly consistent for all samples, indicating that the *cm*PCR is an efficient method for rapid screening of large number of samples. This clearly validates that the *cm*PCR protocol is a very useful and sensitive tool to obtain information on the composition and population dynamics of the specific genera from complex microbial communities. The combined application of multiplex PCR and crude DNA extraction as described here, will allow routine, high-throughput analysis of environmental samples for the quick assessment of potential antagonistic bacteria (*Bacillus* spp. and *Pseudomonas* spp.).

When considering the extensive disease control or ecological studies, focusing on diversity of microbial interactions, simultaneous testing becomes more valuable and effective. The employment of this method may provide a new way to investigate the microbial populations in a wide range of environments in a short span of time. This will further help in finding distribution, abundance, and screening of *Bacillus* spp. and *Pseudomonas* spp. from a large number of environmental sites and to explore its biotechnological potential.

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Conflict of interest All authors declare that they have no conflicts of interest.

References

1. Altinok I (2011) Multiplex PCR assay for detection of four major bacterial pathogens causing rainbow trout disease. Dis Aquat Organ 93(3):199–206
2. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410
3. Balcazar JL, Rojas-Luna T (2007) Inhibitory activity of probiotic *Bacillus subtilis* UTM 126 against *Vibrio* species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). Curr Microbiol 55(5):409–412
4. Bobbie RJ, White DC (1980) Characterization of benthic microbial community structure by high-resolution gas chromatography of fatty acid methyl esters. Appl Environ Microbiol 39(6):1212–1222
5. Chamberlain JS, Chamberlain JR (1994) Optimization of multiplex PCRs. In: Mullis KB, Ferre F, Gibbs RA (eds) The polymerase chain reaction. Birkhauser, Boston
6. Chen J, Tanq J, Liu J, Cai Z, Bai X (2012) Development and evaluation of a multiplex PCR for simultaneous detection of five food borne pathogens. J Appl Microbiol 112(4):823–830
7. Chiang YC, Yang CY, Li C, Ho YC, Lin CK, Tsen HY (2006) Identification of *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. with 16S ribosomal DNA-based oligonucleotide array hybridization. Int J Food Microbiol 107(2):131–137
8. Cunningham CO (2002) Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. Aquaculture 206:19–55
9. FAO (2012) The state of world fisheries and aquaculture 2012. Italy, Rome
10. Fan H, Wu Q, Kou X (2008) Co-detection of five species of water-borne bacteria by multiplex PCR. Life Sci J 5(4):47–54
11. Garbeva P, Veen JA, Elsas JD (2004) Assessment of the diversity, and antagonism towards *Rhizoctonia solani* AG3, of *Pseudomonas* species in soil from different agricultural regimes. FEMS Microbiol Ecol 47(1):51–64
12. Gomez-Gil B, Roque A, Turnbull JF (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191:259–270
13. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment and editor program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98
14. Hofmann MA, Brian DA (1991) Sequencing PCR DNA amplified directly from a bacterial colony. Biotechniques 11(1):30–31
15. Janda JM, Abbott SL (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol 45(9):2761–2764
16. Kong P, Richardson PA, Hong C (2005) Direct colony PCR-SSCP for detection of multiple pythiaceous oomycetes in environmental samples. J Microbiol Methods 61(1):25–32
17. Krieg NR, Holt JG (1984) Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore
18. Kwon HS, Yang EH, Lee SH, Yeon SW, Kang BH, Kim TY (2005) Rapid identification of potentially probiotic *Bifidobacterium* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiol Lett 250:55–62
19. Kwon G-H, Lee H-A, Park J-Y, Kim JS, Lim J, Park C-S, Kwon DY, Kim Y-S, Kim JH (2009) Development of a RAPD-PCR method for identification of *Bacillus* species isolated from Cheonggukjang. Int J Food Microbiol 129:282–287
20. Logan NA, Berkeley RCW (1984) Identification of *Bacillus* strains using the API system. J Gen Microbiol 130(7):1871–1882
21. Maeda Y, Shinohara H, Kiba A, Ohnishi K, Furuya N, Kawamura Y, Ezaki T, Vandamme P, Tsushima S, Hikichi Y (2006) Phylogenetic study and multiplex PCR-based detection of *Burkholderia plantarii*, *Burkholderia glumae* and *Burkholderia gladioli* using gyrB and rpoD sequences. Int J Syst Evol Microbiol 56(Pt 5):1031–1038
22. Nair AV, Vijayan KK, Chakraborty K, Leo Antony M (2012) Diversity and characterization of antagonistic bacteria from tropical estuarine habitats of Cochin, India for fish health management. World J Microbiol Biotechnol 28(7):2581–2592
23. Nithya C, Pandian SK (2010) Isolation of heterotrophic bacteria from Palk Bay sediments showing heavy metal tolerance and antibiotic production. Microbiol Res 165(7):578–593
24. Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol 37(6):1661–1669
25. Ozdemir Z (2009) Development of a multiplex PCR assay for the simultaneous detection of *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas axonopodis* pv. *vesicatoria* using pure cultures. J Plant Pathol 91(2):495–497
26. Perry L, Heard P, Kane M, Kim H, Savikhin S, Dominguez W, Applegate B (2007) Application of multiplex polymerase chain reaction to the detection of pathogens in food. J Rapid Methods Autom Microbiol 15(2):176–198
27. Sadeghi A, Mortazavi SA, Bahrami AR, Sadeghi B (2012) Design of multiplex PCR for simultaneous detection of rope-forming *Bacillus* strains in Iranian bread dough. J Sci Food Agric 92(13):2652–2656
28. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
29. Sharma S, Kaur M (2010) Antimicrobial activities of rhizobacterial strains of *Pseudomonas* and *Bacillus* strains isolated from rhizosphere soil of carnation (*Dianthus caryophyllus* cv. Sunrise). Indian J Microbiol 50:229–232
30. Spilker T, Coenye T, Vandamme P, LiPuma JJ (2004) PCR-based assay for differentiation of *Pseudomonas* aeruginosa from other *Pseudomonas* species recovered from cystic fibrosis patients. J Clin Microbiol 42(5):2074–2079
31. Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44(4):846–849
32. van Zeijl CM, van de Kamp EH, Punt PJ, Selten GC, Hauer B, van Gorcom RF, van den Hondel CA (1998) An improved colony-PCR method for filamentous fungi for amplification of PCR-fragments of several kilobases. J Biotechnol 59(3):221–224
33. Vaerewijck MJ, De Vos P, Lebbe L, Scheldeman P, Hoste B, Heyndrickx M (2001) Occurrence of *Bacillus sporothermodurans* and other aerobic spore-forming species in feed concentrate for dairy cattle. J Appl Microbiol 91(6):1074–1084
34. Vijayan KK, Singh ISB, Jayaprakash NS, Alavandi SV, Pai SS, Preetha R, Rajan JJS, Santiago TC (2006) A brackish water of

Pseudomonas PS-102, a potential antagonistic bacterium against pathogenic vibrios in penaeid and non-penaeid rearing systems. Aquaculture 251(2–4):192–200
35. Vinoj G, Vaseeharan B, Jayaseelan BD, Rajakumaran P, Ravi C (2013) Inhibitory effects of *Bacillus licheniformis* (DAB1) and *Pseudomonas aeruginosa* (DAP1) against *Vibrio parahaemolyticus* isolated from *Fenneropenaeus indicus*. Aquac Int. doi:10.1007/s10499-012-9617-2
36. Wan M, Rosenberg JN, Faruq J, Betenbaugh MJ, Xia J (2011) An improved colony PCR procedure for genetic screening of *Chlorella* and related microalgae. Biotechnol Lett 33(8):1615–1619
37. Ward AC (1992) Rapid analysis of yeast transformants using colony-PCR. Biotechniques 13(3):350
38. Wattiau P, Renard ME, Ledent P, Debois V, Blackman G, Agathos SN (2001) A PCR test to identify *Bacillus subtilis* and closely related species and its application to the monitoring of wastewater biotreatment. Appl Microbiol Biotechnol 56(5–6):816–819
39. Wu X-Y, Walker MJ, Hornitzky M, Chin J (2006) Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance. J Microbiol Methods 64:107–119
40. Zheng L, Han X, Chen H, Lin W, Yan X (2005) Marine bacteria associated with marine macroorganisms: the potential antimicrobial resources. Ann Microbiol 55(2):119–124