Eco. Env. & Cons. 27 (February Suppl. Issue) : 2021; pp. (S381-S385)
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ISSN 0971–765X

Evaluation of primer specificity used for detection and formation of Viable But Non-Culturable forms of *Ralstonia solanacearum*

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(Received 29 July, 2020; Accepted 20 October, 2020)

ABSTRACT

Ralstonia solanacearum is Gram-negative bacterial phyto-pathogens, which cause bacterial wilt disease in several vegetable crops of *Solanaceae* family. The bacterium is mainly soil and water borne in nature and spread through running water between different agricultural fields. In environment it can survive as VBNC (Viable But Not Culturable) state. Reverse Transcriptase qPCR (RT-qPCR) targeting housekeeping genes is a promising and reliable technique used to detect the VBNC forms of the pathogen. In present investigation three housekeeping genes namely, *16S rRNA*, *RpoS*, and *Omp*, were evaluated for their specificity. Further to access the viability of the cells 16srDNA (*16S rRNA*) gene transcript was determined along with pathogenicity test. Strains of *R. solanacearum* DIBER-117 showed a 20% decrease in 16SrDNA gene transcriptome after 480 days. Among the three sets of primers tested, the primer targeting *16s rDNA* is found to have better specificity.

Key words : *VBNC, Culturability, Housekeeping Genes, RT-qPCR*

Introduction

Ralstonia solanacearum is an important Gram negative bacterial phyto-pathogen that causes bacterial wilt disease in solanaceous crops and other ornamental plants (Alvarez and Biosca, 2017). To date, *R. solanacearum* has been reported from more than 400 plant species of 50 different families, and the host range of the pathogen is continuously expanding (Xue *et al*., 2011). More importantly, it has been considered as second most devastating plant pathogenic bacterium (Mansfield *et al*., 2012. In the environment, the pathogen survives as Viable But Non-Culturable (VBNC) state, starved cells and biofilm (Ducret *et al*., 2014). In VBNCs cells don't grow in microbial media but, they remain virulent and can cause disease. The survival of VBNC forms in the environment is influenced by a number of abiotic factors, among them temperature plays most important role. VBNCs can survive up to four years in deeper layers of soil, where temperature fluctuation is minimal. Importantly, VBNCs can survive up to four or more years without losing its pathogenicity (Alvarez *et al*., 2008). Although, for *R. solanacearum*

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diagnosis various detection methods including traditional culture methods and PCR assays are available (Pastrik and Maiss, 2000; Perera *et al*., 2018). VBNCs can be undiagnosed with these detection techniques. *R. solanacearum* maintains a minimal expression of some essential housekeeping genes associated with bacterial survival and stress response *viz. 16S rRNA, Omp* and *RpoS* VBNC detection (Kong *et al*., 2014). Genes like *16S rRNA* synthesis are continuously expressed during this state, *RpoS known* as Stress Regulator protein which is involved in regulating the expression of genes associated with bacterial survival and stress response. In addition to that, *RpoS* genes are involved in the production of antioxidant enzymes. *Omp* gene is an outer membrane protein which is produced constitutively in both culturable and VBNC cells (Kong *et al*., 2004). In this work, the specificity of primers targeting housekeeping has been evaluated with the aim to detect VBNC Form of *R. solanacearum* from water and mixed environmental samples. This finding will have a direct implication on devising dete ction strategies of VBNCs cell

Material and Methods

Bacterial strains and culture medium

R. solanacearum strains DIBER 115 (Accession number-MG266193), DIBER 116 (Accession number - MG266202), DIBER 117 (Accession number-MG266199) and DIBER 118 (Accession number-MG266203) used in the present study were isolated from the diseased tomato plants of different location of Uttarakhand, India. The strains were confirmed by 16S rDNA sequencing and Multiplex PCR (Opina *et al*., 1997). In addition, *R. solanacearum strains* Rs0418 was procured from (National Agriculturally Important Microbial Cultu re collection, (NAIMCC) *E. coli* (ATCC® 10536™), *Bacillus cereus* (ATCC ®11778™), *Pseudomonas* sp. (DIBER 114), *Ralstonia pickettii* (ATCC® 648™), *Ralstonia insididosa* (ATCC49129™) and *Ochrobacterium* sp. (DIBER-102) were used for the study of specificity of PCR.

Primer designing and evaluation of primer specificity

To develop a RT-qPCR assay, we have targeted three housekeeping genes namely, *16S rRNA, RpoS,* and *Omp*. Primers were designed against the conserved sequences of respective genes from 40 strains of *R. solanacearum* that were retrieved from NCBI. The sequences were aligned using CLC-sequence viewer and primers were designed from the conserved regions of respective genes. The specificity of the *16S rRNA, RpoS*, and *Omp* primers designed in this study which is mention in Table 1. were evaluated using DNA extracted from *R. solanacearum* and non *R. solanacearum* strain including *R. pickettii*, *R. insididosa, Escherichia coli*, *Bacillus cereus*, *Pseudomonas Sp*., *Ochrobacterium sp*. and metagenomic DNA from soil. The concentrations of all the DNA templates were normalized to 50 ng/µL for the PCR amplification. PCR were performed in the Q-Cycler 96+, Hain Lifesciences, UK. Thereafter, amplified PCR products were resolved in 2% agarose gel.

Studies on the formation of VBNC state of *R. solanacearum* in water

VBNC cells of *R. solanacearum (*DIBER 117) were prepared by incubating water washed cells 10^8 CFU/ml at room temperature (Alvarez *et al.,* 2008). Their survival was periodically monitored at 0, 60, 120, 180, 240, 300, 360, 420 and 480 days by direct viable count in mSMSA media was performed as follows. The cell pellet was resuspended in 1ml of sterile distilled water and serially diluted using sterile water. 100µl of the dilutions were spread plated on mSMSA agar plates and the resultant colonies were counted and expressed in logarithmic numbers.

Resuscitation of microcosms

Reverse culturability otherwise known as resuscitation is used as an important tool for VBNC confirmation. Briefly, VBNC cells of *R. solanacearum* (DIBER 117) were resuscitated in sterile distilled water supplemented with 1000 U/mL of catalase (HiMedia Labs, Mumbai). The process was carried

Table 1. Primers used in the present study were listed.

Gene	Sequence	Ta (°C)
Rp 16S F/R	5′-CCTGGCTCAGATTGAACGCT-3′ 3′-CTCCTATAGCATGAGGCCTT-5′	62.5
Rp RpoS F/R	5′ACTTCCGCACGGTGTTGCAG-3′ 3′TTGCGCTCGATCATCACCTG-5′	64.4
Rp Omp F/R	5′GAAAATGAAACTGTTTGCAG-3′ 3′CCACGCAGGCCCCAGCGCGA-5′	65.5

at 28°C for five days and the resultant growth was counted on mSMSA plates after proper washing with sterile distilled water (Kong *et al.,* 2004).

Pathogenicity Assays

A pathogenicity assay was performed on bacterial wilt susceptible tomato variety seeds ICAR H-86 as per the procedure of (Singh *et al.,* 2018). Briefly, five seedlings of tomato were challenged at two leaf stage by root dip method by inoculating in 1 mL of *R. solanacearum* for 10 min. Plants were air dried for 5 min and then kept in centrifuge tubes containing 1.5 mL of sterile water. Further, plants were maintained in a controlled environment with 65-70% humidity and temperature of 30± 1° C for 7 days. The disease severity scale was determined on 7th day according to the key; 0 = No symptoms, 1= One leaf wilted, 2 = Two leaves wilted, 3= Plant dead. The Disease index (DI) was calculated by the formula DI=ΣRT/4N, where; R = Disease severity scale, T = Number of wilted plants in each category and N = Total number of tested plants in Fig 2. Pathogenicity index percentages were also mention. Infectivity assays were performed using ICT strips (Adgia, USA) Fig. 2.

RT-qPCR

The washed cells were incubated at Room temperature RT-qPCR was performed using the primer set Rp16F (5'-CCTGG CTCAGATTGAACGCT-3') and Rp16R (3'-CTCCTATAGCATGAGGCCTT-5') designed against the conserved sequences of *16S rRNA* gene of *R. solanacearum* species complex. The resulting 210 bp PCR product of the above primers were cloned in plasmid pTZ57 R/T (Thermo Fisher) as per the manufacturer's instructions. After extraction and subsequent quantification, 10-fold serial dilutions of the cloned plasmid were made and used as standard for quantifying 16S rRNA (Jain *et al.,* 2012). The RT-qPCR reaction was performed and the results were analysed in CFX96 Real-Time System and CFX manager respectively (Bio-Rad, Hercules, CA, USA). In Fig 2. Data presented as mean±standard deviation (SD) of replicates.

Results

Evaluation of Primer Specificity

In the present study, tested strains belong to Phylotype I of *R. solanacearum* species complex (Prior P, 2005, Safini *et al*., 2014). Importanty primers including *16SrRNA*, *Omp,* and *RpoS, are* mostly used for VBNC detection and are in-silico validated for their matches/mismatches in their respective genes. For each gene no mismatches found in our primers. The primers Rp *RpoS* F & R primer gave non-specific amplification with other tested Bacterial strains such as *R. picketti*, *R. insidiosa* and *Ochrobactrum* sp. While the primer Rp *Omp* F & R was not efficient in amplifying the sequences as numerous nonspecific amplicons are formed with all the tested strains. The primers Rp *16s* F & R gave non- specific amplification only in metagenomic DNA.

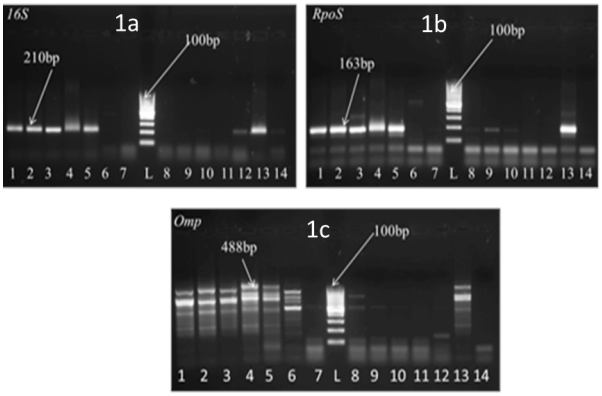

Fig. 1. **1a.** *16S rRNA*, **1b**. *RpoS,* **1c**.*Omp* :1- DIBER 117; 2-DIBER 115; 3-DIBER 118; 4-DIBER 116; 5-0418; 6-*Escherichia coli*; 7 *Bacillus cereus*; L- Ladder 100 bp; 8-*Pseudomonas Sp.*; 9-*Ralstonia pickettii*; 10-*Rasltonia insididosa*; 11-*Ochrobacteri um sp*; 12-Soil DNA without *R. Solanace arum* and 13- Positive control and 14- Non template control.

Confirmation of VBNC state in *R. solanacearum*

For evaluating the formation of Viable But Non Culturable Form of *R. solanacearum* out of four tested strains, *R. solanacearum* strains DIBER117 choosen for this experiment for the study of VBNC. DIBER117 in Room temperature lost their culturability within 480 days as determined by direct plate counting. In present investigation reverse culturability of the strain DIBER117 was continuously maintained. To examine VBNC state resuscitation of microcosm was investigat ed using catalase and CFU count was monitored after 5 days of incubation. We found that DIBER 117 showed 50% reverse culturability after 480 days. To access the viability of the cells 16srDNA gene transcript was determine along with pathogenicity test. DIBER117

showed a 20% decrease in 16srdna gene transcriptome after 480days of incubation. Further to examine the wiliting symptoms pathogenicity index was recorded, DIBER 117 showed 90% diseases index at 480 days Fig 2. and Fig 3. Showing confirmati on on of pathogenicity test by ICT strips.

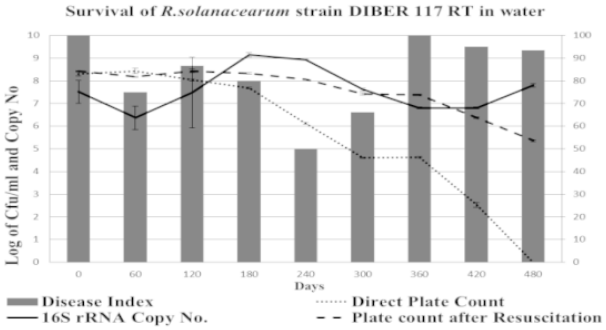

Fig. 2. Survival of *R. solanacearum* strain DIBER 117 in spiked water.

Fig. 3. Results showing Pathogenicity test of *R. solanacearum* strain (DIBER 117). Control plants ICT strip test negative e and Test plants ICT strip test positive.

Discussion

The regulatory genes *16s* rRNA, *Omp,* and *RpoS are* likely to be important for the induction of VBNC state detection. It is well known that cells which are in VBNC state are metabolically active and can synthesize mRNA molecules. mRNA has been proposed as a good viability marker because of its central role in cell metabolism as well as its very short half-life. The results of present investigations are in harmony as been reported earlier that RT-qPCR is a promising methods for the detection of variety of genes in VBNC state (Lahtinen, *et al*., 2008). Before using these targets for future endeavours related to VBNCs, we evaluated the specificity of the primers targeting them. Among the three primers selected, the primer Rp *16s* F & R is found better that the other two primer sets, hence can be used for future studies. The resuscitation consider as a fundamental property of VBNC forms (Kim *et al.*, 2018) and for confirmation of resuscitation, catalase (Zhao X *et al*., 2017) is consider a good method of reverse culturability, our results also follow the same direction as been reported by several researchers earlier. Our results regarding the culturability and pathogenicity were not similar with the experimental results (Alvarez *et al.*, 2008). Who reported that *R. solanacearum maintained* VBNC state for 1460 days i.e up to 4 years, the reason for such variation can be attributed to be difference of phlotype, and physiological conditions (Ramamurthy *et al*., 2014). The knowledge about the pathogenicity and molecular control mechanism of VBNC state will help us to know the physiology that will ultimately provide some new information for developing novel detection and/or control methods for VBNC forming bacteria.

Conclusion

VBNC cells in mixed populations were constrained due to the non-specificity of the tested primers for confirming the live/dead status of cells. In future its mandatary to evaluate and validate more specific transcriptome targets for VBNC detection.

Equal Contribution

Neha Faridi and Shalini Bhatt contributed equally in the manuscript and thus both consider as lead author

Acknowledgements

The authors wish to express their sincere thanks to Director DIBER, DRDO Haldwani and Dr. Veena Pande, Co-ordi nator, Department of Biotechnology, Bh imtal, Kumaun University, Nainital, India for providing the facilities, support and encouragement. Dr. Ravindra Chhimwal and Dr. Tahir Sidqqui for moral support

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