

Original Research Article

DOI - 10.26479/2017.0301.03

## THE VALIDITY OF SINGLEPLEX AND MULTIPLEX REAL TIME PCR DETECTION AND QUANTIFICATION OF WATERBORNE PATHOGENS FROM DOMESTIC TO INDUSTRIAL WATER

Ousman Bajinka, Guven Ozdemir

General and Industrial Microbiology, Ege University, Izmir Turkey.

**ABSTRACT:** Isolation, detection and quantification of pathogenic DNA is the novel approach when it comes to the diagnosis of infectious disease. In every laboratory more especially in the waterborne pathogenic research laboratories, achieving an accurate result is always the main goal. Efficient and reliable results are not achieved using only the old conventional methods due to number of reasons. To this end, molecular methods that include real-time polymerase chain reactions (real-time PCRs) for the detection of nucleic acid stand novel. In this review, the efficiency, sensitivity and reliability of singleplex and multiplex real-time PCR are thoroughly considered. Further analysis on their contrasting validities was also briefed on. Articles were randomly selected with some specificity to the previous water microbiology studies. From the review, it is clear that both the use of singleplex and multiplex real-time PCR are always valid for the waterborne pathogen detection. However, majority of the researchers would prefer to choose multiplex over singleplex. Both has shown a considerable validity but running for multiple target in single reaction tube assured both the economic cost and saves time.

**KEYWORDS:** Real-Time Polymerase Chain Reaction, Waterborne Pathogens, Efficiency, Sensitivity and Reliability

**\*Corresponding Author: Ousman Bajinka**

General and Industrial Microbiology, Ege University, Izmir Turkey

\* Email Address: bajinkaousman@gmail.com

© 2017 Life Science Informatics Publication All rights reserved

Peer review under responsibility of Life Science Informatics Publications

2017 May- June RJLBPCS 3(1) Page No.25

## 1. INTRODUCTION

Water and wastewater microbiology has been a discipline of great interest since the birth of microbiology (Outbreak of cholera). However, the perception of winning the battle against waterborne pathogen has been inculcated in the minds of many, more especially the developed world who has done a considerable effort in attaining high standards of used water. Due to this, the re-emergence of old won battle is once more becoming a new battle. Morbidity and mortality rate are caused by infectious diseases that includes diarrhea is a global diseases burden. It is estimated that 4% deaths globally are caused by diarrhea and 5.7% of global diseases are as a result of waterborne infection and water related illness [1]. It is reported that waterborne diseases cause more than 2.2 million deaths per year and among these, bacterial, viral, parasitic, schistosomiasis, trachoma, ascariasis, and trichuriasis are on the list of the diseases [2]. Gastrointestinal diseases and some systematic illness are the frequent occurrence in the hospitals of underdeveloped world due to the poor facilities and sanitation approaches [2, 3]. Among the infectious agents are bacteria, protozoa, viruses and helminths, and this can be contracted by either ingestion, contact with contaminated water or airborne [2]. In response to United Nations' consideration of providing an improved water quality and safe for drinking by 50% in 2015 as part of the eight Millennium Development Goals (MDGs), the preservation and creating the ways of using clean and safe water is inexplicable [2,4]. Among the agents that can contaminate our water, are chemical, physical and microbiological and these are threats to our health or health related problems. In addition, external factors that are environmental including; temperature, salinity and nutrient enrichment are link to the abundance and occurrence of pathogenic microbes in water bodies[4,5] Tracking down the exact sources of water contaminants requires both considering chemical and microbial agents and these two are interwoven and should not precede without the other. [4] It is crystal clear that among the sources of microbiological contamination are those microbes from human or animal excreta and the washing away of these excreta to our primary source of water enable us to be living with numerous health problems. [2,4,5] In addition to the washed waste, the leaked pipes that serve us the usable water from the primary source to the different distributions sometimes enable the formation of biofilms that can contained human pathogens. [1,2] The causative agents for the very most common outbreaks are but not limited to, *Campylobacter*, *Salmonella*, *Shigella*, *Escherichia coli* (*E.coli* O157), *Cryptosporidium*, *Giardia* and *Norovirus* [2, 5]. Chlorine has been and still in use as a means to disinfect water against pathogens however, agents like protozoans and some viruses have already built resistance against chlorine compounds [15]. For accurate detection of these waterborne pathogens that are a threat to our health, methods more than a mere indicator approach are required.

Traditionally, the conventional approach of the use of indicator organisms are much relied on to an extent that the mere absent or missing the occurrence of *E. coli* (using fecal coliform test) in a water sample determines it safe (this is an outdated approach). Out of no doubt, there are some waterborne pathogens other than indicator parameters and these includes viable but non-cultural organisms, meaning the pathogenic agent is active but cannot be cultured [6]. A more reliable, rapid and methods that will hardly miss out pathogenic agents are vital. [9, 10] In the recent years, Nucleic Acid Tests (NATs) are among the leading molecular techniques for the identification of waterborne and water related pathogens. These methods are based on the affinity of specific nucleic acid sequences and they produce highly specific reaction results [8, 11] The most frequently used techniques are based on Polymerase Chain Reaction (PCR) methods, isothermal nucleic acid amplification or microarrays. Among these NATs, PCR still stood distinct. On top of its ability in rapidly detecting pathogenic DNA, PCR tests are also highly sensitive and specific [8, 11, 12, 13]. At some instance PCRs have some similarities with microbial growth or replication however, while for microbial replication; all the cells are involved in the microbial growth, only a targeted template of DNA is been amplified in PCRs [2, 14] Conventional PCR was the method that is used to amplify DNA and analysis using gel electrophoresis to separates using base pair (bp). Since the coming of real-time PCR in the science of molecular methods, the old and time consuming post amplification analysis (conventional PCR) is a thing of a past [1,2]. Microfluidic digital PCR (RT-dPCR) is among the most successful recent developed Nucleic Acid Technologies (NATs).It is an absolute quantification approach that has a novel precision by producing a very accurate endpoint-sensitive even without the introduction of standard curve as in real-time PCR, it can accurately determine the number of target copies [16]

Figure 1; Polymerase Change Reaction (PCR) Conditions

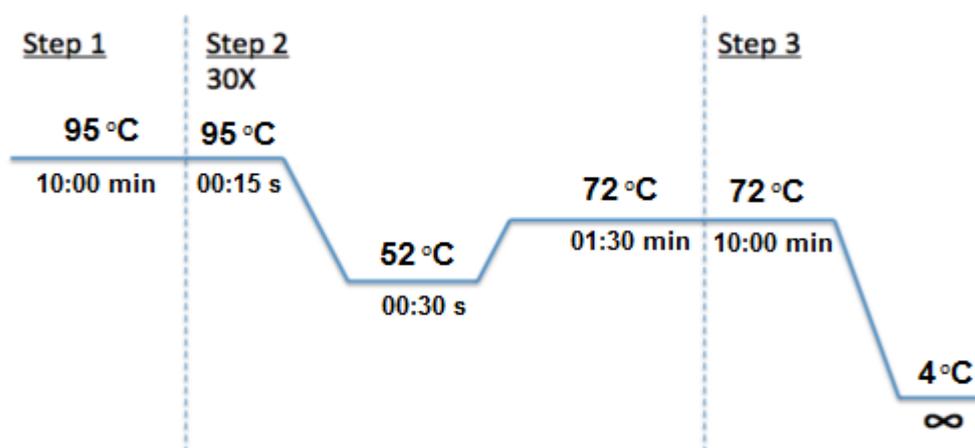


Figure 1: PCR conditions for three hypervariable regions of mtDNA

The normal PCR reaction master mix contains Water, 10x Reaction Buffer, MgCl<sub>2</sub>, 2 dNTPs, Forward Primer, Reverse Primer, Target DNA, Polymerase enzyme. ©2005 and 2011 Integrated DNA Technologies. All rights reserved.

### **Quantitative real-time PCR (qPCR)**

In a bid to combat the laborious time that we spend on the post amplification using gel electrophoresis, to increase both sensitivity and specificity while paying more attention to the reliability of the test result, real time Polymerase Chain Reaction (real time-PCR) was required [8,13,15]. Real time PCR is a very powerful and rapid nucleic acid amplifying devices that is also simple to use. [14,15] It operates as the usual principles of PCR and it has an additional advantage of the ability to amplify DNA while the reaction is still in progress hence its name 'real-time PCR' and it gives a highly specific detection with the help of sequence-specific DNA probe (This consists 8 oligonucleotides labeled with a fluorescent reporter) [8,15]. Despite the inability to detect RNA by PCR, a reverse transcription step (RT-qPCR) is always added to form a complimentary DNA (cDNA) that can be both detected and quantified. [8]. The simultaneous analysis and collecting data can be achieved with real time PCR reactions. This simply means that both the amplification and analysis occur together. [13,14,15] In contrast to the conventional PCR, either Deoxyribonucleic acid (DNA) dyes or the fluorescent probes are among the reagents mixture added before running the machine. Since there is no need to transfer samples from a thermocycler to gel electrophoresis reading, (Samples are automatically analyzed and the results are shown on the computer screen). Moreover, this will ensure the low level of risk associated with contamination that would likely affect the amplicon (amplification product). [14] Like any other methods of detection and quantification, real time PCR has some of its limitations that will more likely alter the amplicon size during the reaction however, the powerful melting curve analysis are employed to verify the sample product amplified and further detect the sequence variants up to the single base [2, 14]. The temperature cycling is used to amplify the specific target of the molecule of DNA, and the amplification is recorded in each cycle number. Either the DNA binding dyes; SYBR Green 1 or sequence specific probes; TaqMan, Molecular Beacon is used to detect amplification [14]. PCR efficiency is a powerful tool that is used to control and monitor the growth rate. And this depends mainly on the primers and temperature cycling conditions. If the starting amount of samples and the PCR efficiency are known, a precise quantification is assured [14]. Sensitivity is the ability to detect the smallest of amount of DNA, cDNA or RNA for a reaction and serial dilution (e.g. 2 X 10<sup>2</sup> to 2 X 10<sup>10</sup>) is used to determine the sensitivity of PCR assay [13]. qPCR has achieved a milestone in the field of water monitoring. There are very specific kits designed for the detection of specific

waterborne pathogen. Examples can be iQ-Check Legionella Real-Time PCR kits (from BioRad; AquaScreen® qPCR kits ) for quantitative detection of Legionella pneumophila, Legionella species, Pseudomonas aeruginosa and Escherichia coli from Minerva Biolabs GmbH; Enterovirus Real Time PCR kit from Diagenode, and so on [10] Recently, a nanofluidic real-time PCR (RT-qPCR) system has an efficient ability to detect waterborne human pathogenic viruses. [16] Comparing conventional real-time PCR system to the Microfluidic digital PCR (RT-dPCR) and RT-qPCR for detection and quantification of human pathogenic viruses can be found in Table 1

Name of Test	Test Used For	Efficiency	Limitations	Reference
Multiplex -real time PCR	Detection of nosocomial pathogens in clinical samples	The identification efficiency is 97% (43 out of 46 isolates were positive)	The other 3 isolates were beyond the scope of identification due to the limited primers selected	Anbazhagan D et al (2011)
Multiplex conventional PCR	Multiplex PCR Assay for Detection of bacterial Pathogens Associated with Warm-Water Streptococcosis in Fish	The sensitivity of multiplex PCR assay were 25 pg for <i>S.iniae</i> , 12.5pg for <i>S.difficilis</i> , 30pg for <i>S.parauberis</i> and 50pg for <i>L.garvieae</i>	Although some multiplex PCRs shows limits of detection, they were run in triplicate.	Mata A.I et al (2003)
microfluidic qPCR (multiplex)	simultaneous quantification of multiple food- and waterborne pathogens	It overcomes multiple pathogens of concern, multiple samples to be analyzed during routine monitoring, and generally low concentrations of pathogens.	It failed to detect <i>L. monocytogenes</i> serovar 4c strain JCM 7679 by <i>iap</i> and <i>hlyA</i> -targeted qPCR, (false negatives) most likely due to the mismatches present in the primer annealing sites.	Ishii H et al (2013).
Real-Time PCR method	For the presence ( <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Pseudomonas aeruginosa</i> and <i>Aeromonas hydrophila</i> ) in both particulate matter (PM) and in rainwater (RW)	These pathogens were found to be prevalent in both PM and RW samples with <i>E. coli</i> being the most prevalent potential pathogen in both types of samples. This shows a high sensitivity.	The atmospheric conditions might have an effect on the number of expected pathogens	<u>Rajni Kaushik</u> R et al (2012)

Multiplex PCR	Molecular detection of coliforms and shorten the time of PCR.	PCR results sensitivity 86% (95% CI: 0.71-0.93). In addition, the total execution time, with a successful change of factors, was reduced to less than two and a half hour.	It's recommended to be used at least as an initial screening test, and then the positive samples could be randomly tested by MPN. Henceforth time is really consumed.	<u>Fatemeh D et al</u> (2014)
nanofluidic real-time PCR (RT-qPCR)	Comparing conventional real-time PCR system to the RT-dPCR and RT-qPCR for detection and quantification of human pathogenic viruses	Sensitivity as observed and recorded was slightly higher in RT-qPCR than with RT-dPCR for 14 viruses by a factor range of from 0.3 to 1.6 log <sub>10</sub> .	Both the assays are successful though RT-dPCR will show some slight sensitive over the RT-qPCR	Coudry-Meunier C et al (2015).
TaqMan <sup>®</sup> real-time quantitative PCR (qPCR).	<i>Test for the presence of Aeromonas hydrophila, Bacillus cereus, Clostridium perfringens, Enterococcus faecalis, Escherichia coli, E. coli O157:H7, Helicobacter pylori, Klebsiella pneumoniae, Legionella pneumophila, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella sp., and Staphylococcus aureus.</i>	Effective for the detection of pathogens in wastewater and confirmed that the risk of exposure to pathogens in the wastewater discharge.		<u>K.E. Shannon et al</u> (2007)

Figure 2: Reverse Transcriptase Real-Time PCR. Zhang X et al (2015)

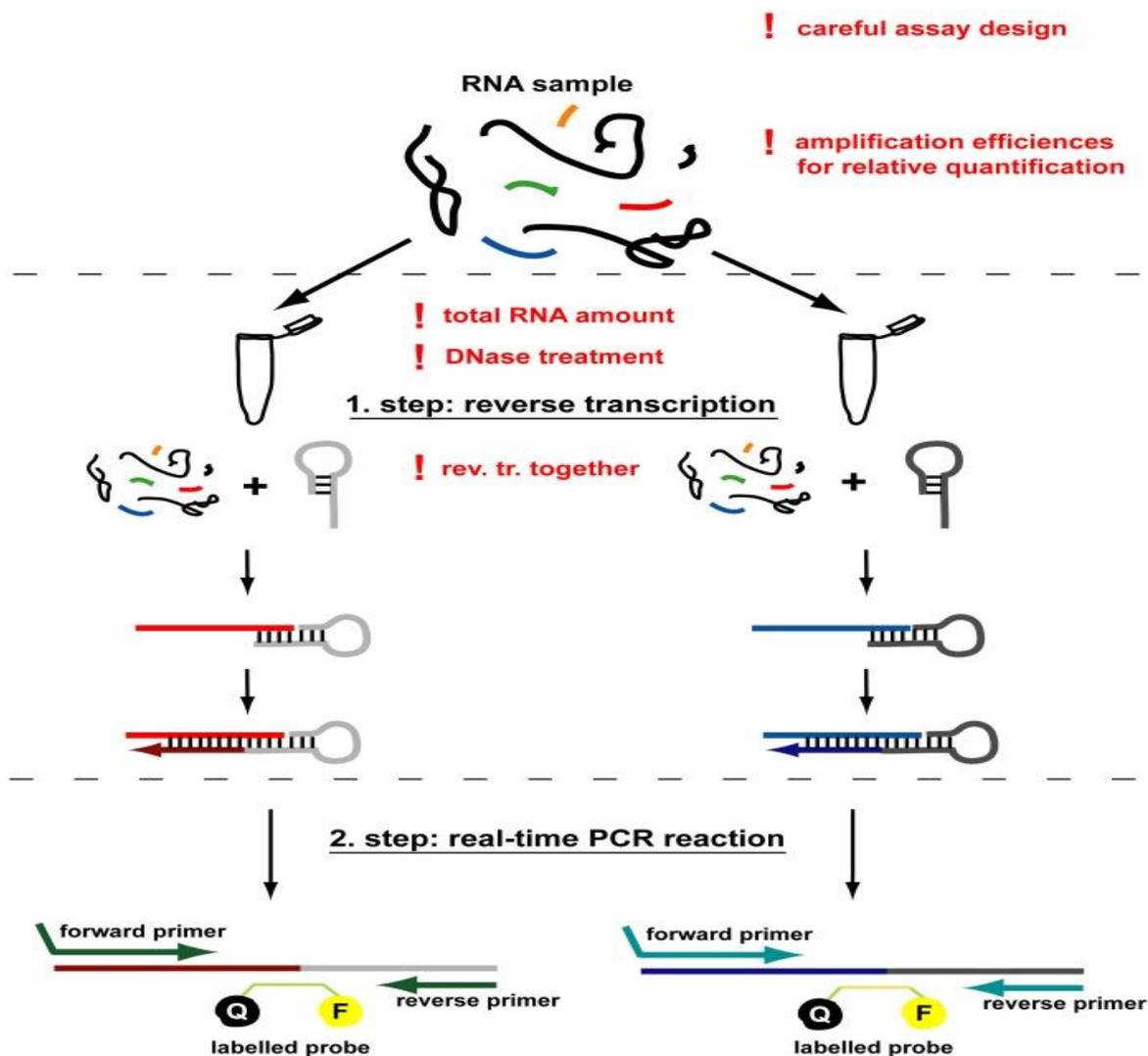


Figure 2: In reverse transcription and real-time PCR, it involves two steps of which sometimes these are carried out simultaneously. However, in this figure a separate step are shown. ‘In the first step, mature miRNA is extended and reverse transcribed by a sequence specific stem-loop primer. In the second step, a fluorescently labeled hybridization probe using the strand replacement reaction quantifies the reverse transcribed miRNA. According to the previous protocol, all targets (e.g. endogenous control and target) should be reverse transcribed separately. In the dual-labeled probe based detection systems Q stands for quencher, F for fluorophore. Red exclamation marks indicate crucial points of the procedure that are discussed in this paper’. The read marks are indicating critical points during reaction.

## **Multiplex PCR**

The desire for accurate results in short time is of no doubt very high. The detection of single pathogen of different strains or the combination of different species in a single reaction tube is extremely inexplicable. The simultaneous detection of pathogens is both cost-effective and time economic [17]. With the simultaneous detection of multiple pathogens, other molecular methods other than multiplex PCR are used. These includes but not limited to, pyrosequencing (a method that relies on pyrophosphate release on nucleotide incorporation to determine the order of nucleotides in DNA), microarray hybridization (an assay that hybridizes mRNA molecule to the original DNA template) and other hybridization detection methods [17]. However, when compared these with multiplex real-time PCR, quantitative information and more sensitivity is likely to be obtained by multiplex real-time PCR. For example concerning their specificity, some pathogens are more likely unable to differentiate from a non-pathogenic based on their 16S rRNA gene sequence. [7] Despite the limitation of the number of different fluorophores (TaqMan Probe labeled with different fluorophores) that can be used to amplify multiple pathogens in a single tube, at least detection of six different *E.coli* strains is far more preferred to singleplex where only pathogen at a time will be detected. [7] Microfluidic digital PCR (RT-dPCR) is among the most successful recent developed Nucleic Acid Technologies (NATs).It is an absolute quantification approach that has a novel precision by producing a very accurate endpoint-sensitive even without the introduction of standard curve as in real-time PCR, it can accurately determine the number of target copies [16]

## **4. CONCLUSION**

Although polymerase change reaction (PCR) are likely to missed out viable but non-culture organisms (*E.coli*, *Helicobacter* and *V. cholera*), combining it with the culture methods can help to ensure the purity of the DNA to be amplified and hence decreasing the chance of having both false negative and the false positive results. [14] The utilization of one specific primer pair per gene detection reaction can be a huddle, but a proper primer design can iron out these kinks. Another limitation is in the case of multiplex PCR; despite the successful combination of reactions in one tube, they rarely exceed more than six primer sets because the non-specificity and false positivity of a test is likely to occur. [12]

## **CONFLICT OF INTEREST**

The authors have no conflict of interest.

## REFERENCES

1. K.E. Shannon et al. (2007) Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. *Science of the Total Environment*.383(1), 121-129.
2. Yazmin Ramirez-Castillo F et al (2015). Waterborne Pathogens: Detection Methods and Challenges. *Pathogens* 4, 307-334
3. Golberg A et al. (2014) Cloud-Enabled Microscopy and Droplet Microfluidic Platform for Specific Detection of *Escherichia coli* in Water. *PLoS ONE* 9(1): e86341
4. Mostafa H.A et al (2013) Microbiological and Physicochemical Evaluation of Groundwater in Egypt. *International Journal of Environment and Sustainability*. 2(2) 1-10
5. Laczka OF et al (2014) Surface Immuno-Functionalization for the capture and Detection of *Vibrio* Species in the Marine Environment: A New Management Tool for Industrial Facilities. *PLoS One* 9(10): e108387
6. Fatemeh D et al (2014) Rapid detection of coliforms in drinking water of Arak city using multiplex PCR method in comparison with the standard method of culture (Most Probably Number) *Asian Pac J Trop Biomed*. 4(5): 404–409.
7. Ishii H et al (2013). Simultaneous Quantification of Multiple Food- and Waterborne Pathogens by Use of Microfluidic Quantitative PCR. *Appl Environ Microbiol.* ; 79(9): 2891–2898
8. Gall AM, et al. (2015) Waterborne Viruses: A Barrier to Safe Drinking Water. *PLoS Pathog* 11(6): e1004867
9. K.E. Shannon et al. (2007) Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. *Science of The Total Environment*.383(1), 121-129.
10. Valérie Tanchou, CEA (2014) Review of methods for the rapid identification of pathogens in water samples. ERNCIP thematic area Chemical & Biological Risks in the Water Sector Task 7, deliverable 1.Report EUR 26881 EN
11. Morlan J et al. (2009) Mutation Detection by Real-Time PCR: A Simple, Robust and Highly Selective Method. *PLoS ONE* 4(2): e4584.

12. Maynard C M et al (2005) Waterborne Pathogen Detection by Use of Oligonucleotide-Based. applied and environmental microbiology. 71(12), 8548–8557
13. Anbazhagan D et al (2011) Development Of Conventional And Real-Time PCR Assays For The Detection Of Nosocomial Pathogens. Brazilian Journal of Microbiology 42:448-458
14. David H.Persing et al (2011) Molecular Microbiology: diagnostic Principle and Practice, 2<sup>nd</sup> Ed. Edited by ASM press, Washington, DC
15. Dixit U et al (2009) Detection of water-borne pathogens: culture plate to genomics. Indian Journal of Science and Technology. 2(11), ISSN: 0974-6846
16. Coudry-Meunier C et al (2015). A Novel High-Throughput Method for Molecular Detection of Human Pathogenic Viruses Using a Nanofluidic Real-Time PCR System. PLoS ONE 11(1):e0147832
17. Mata A.I et al (2003) Multiplex PCR Assay for Detection of bacterial Pathogens Associated with Warm-Water Streptococcosis in Fish 70(5) 3183-3187
18. Leifels M et al (2016) From Lab to lake-Evaluation of current Molecular Methods for the Detection of Infectious Enteric Viruses in Complex Water Matrices in an Urban Area. PLoS ONE 11(11): e0167105
19. Zhang X et al (2015) Conventional miRNA Detection Strategies. In book: MicroRNA Detection and Pathological Functions, pp.23-35