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Review Article

The Inconsistences of Quantitative Real Time Polymerase Chain Reaction in Diagnostics Microbiology

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Abstract

The use of quantitative methods of real time polymerase chain reactions (PCR) in the amplification and sequencing of DNA and RNA (cDNA) has been a breakthrough in the annals of molecular biology. Its efficiency overweight those of its molecular method counterparts however, against the lights of its specificity and sensitivity there are numerous loopholes that needed to be looked into, analyzed and hence amended for better efficient results. This review highlighted the most common pitfalls and elaborated more on each of them while suggesting ways of improving so as to avert the menace. Articles and research papers were selected at random while making preferences on the most recent papers. Among the inconsistences of quantitative real time PCR; contamination comes in different forms, poor master mix selection and preparations, the impurity of nucleic acid, the cost involved, the inability to detect non-viable from living cells and new emerging cells, poor storing of the specimens and the high technical know-how. In addition, issues regarding multiplex PCR, failure for proofreading and internal controls and the inefficiency of antimicrobial sensitivity tests. All the above pitfalls are avoidable and with the increasing innovation, the future lays the hope of achieving the very best results.

Keywords: Quantitative Real Time PCR; Inconsistences; Efficiency and Reliability; Sensitivity and Specificity

Introduction

It was until the beginning of the last 2 decades that Kary Mullis broke the silence among the scientists with the revolution of even more sophisticated device into our diagnostics laboratories. Before this invention, the diagnostics tools were very limited to conventional methods and these had created our inability to detect many viable but non-culturable pathogens. These days, both of *Norovirus* and *Treponema pallidum* can be diagnosed in the clinical laboratories without the use of electron microscope (EM) and culture plate methods respectively [1,2].

Polymerase Chain Reaction is a deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) in a form of complimentary DNA (cDNA) amplification machine that can detect even the smallest amount of DNA present in the targeted cell or organism. The machine works with the principles of a thermostable DNA polymerase and this eliminate the use of olden system of water bath. *Thermus aquaticus* is a bacterium that can withstand the heat that provides the optimum temperature for the double stranded DNA to be unwound or denature into single stranded and be ready for the alignment of the nucleotide base sequences. The sensitivity and specificity of these methods laid its foundation and made it winning the upper hand [1-4].

With the advent of series of PCR types that includes the quantitative real time PCR, the sectors such as food engineering, diagnostics laboratories (both clinics and research), forensic, genetics and infectious diseases, agricultural production, human genome sequencing projects and the list continues all experienced an overwhelming improvement. The old problematic adage that goes with the hypothesis that; drug-resistant tuberculosis or ventilator-associated pneumonia was difficult to be detected has surrendered to the quantitative PCR and in a real time [5]. In addition to discriminating DNA, in microarray research sectors and qPCR has been widely used for validating expression of miRNAs in whole genome analyses [6,7].

Real time here implies that even during the reaction, the amplification and analysis as well is ongoing. This is the simultaneous advantages found in real time over conventional PCR that will only enable end product analysis by gel electrophoresis. While the conventional PCR relied on the end point analysis can be analyzed based on the base pair (bp) seen on the agar gel electrophoresis, real time PCR has its reaction on amplifying and analyzing the amplified products (amplicon) in real time. Real time PCR confers the simultaneous detection and quantification of the targets that are designed to be read [3,8].

In recent days; the mainstay of medical management and control of diseases (epidemiology) is in the hands of quantitative PCR. The detection of chronic viral infections and fastidious organisms and the accurate detection of an inflammatory bowel disease and bacterial vaginosis brought even more confidence to the research scientists [4,5].

The need for clinical samples to be diagnosed using real time PCR

The prolong culturing and sub culturing that will only obtained its results after the respective biochemical tests is laborious and time consuming. The patients are either going home and turn around for the results or pay for night stays before getting treat-

ment based on the results obtained after days. The introduction of real time PCR has drastically shortened this waiting time from days to hours. The sensitivity (ability to detect even the smallest amount of DNA) and specificity (the ability to detect only the target sequences or organism) of nucleic acid-based tests gain its popularity and the need to established concrete diagnostics [1,5]. The sooner the establishment of the diagnostics results, the better for the treatment and the lesser the patients prone to contamination. Once the wards or laboratories are piled up with patients and specimens respectively, the prevalence of healthcare and hospital acquired infections are high. However, these conditions are well improved on with the introduction of real time PCR in the laboratories [1,4]. Since it came to the light that there are series of pathogenic microbes that are unable to grow or culture in the labs (the appropriate nutritive requirements are yet to be known), the idea of growing for colonies to give results is almost substituted with the non-culture detectable methods conferred by real time PCR. Even the slow growing *Mycobacterium tuberculosis* can now be detected and its risk of specimen treatment has reduced [1,2]. Another reason why real time PCR will still gain popularity in our laboratories is its ability to carry out antimicrobial susceptibility tests [2,9]. In the diagnosis of visceral *leishmaniasis*, real time PCR is sensitive even for immunocompromized and immunocompetent patients [10].

The Reasons for the inconsistency of the results gained by quantitative real time PCR Contaminations

Contaminations sources come in number and vary in the level of contaminating and hence affecting the results of our reactions. In real time PCR, we excelled in the minimization of the contaminants that would have been resulted if convectional PCR was used (real time PCR is carried in a single reaction tube system). However, we are still with the battle against the carryover contaminants. In real time, just as in any other tube system of amplification has number of risks as to the reactions coming in contact with sample residues left not clean prior to the running of the new cycle reactions. The commensals that are often found either on the patients or health care provider or better still environmental aerosols may affect the amplifying of the targeted organism and may lead to false positive results. In addition, these foreign DNAs or particles can as well inhibit the amplification and hence the targeted cells would not be amplified. Human error during the pipetting contributes in no small ways as to the alteration of the results (the negligence of not following aseptic methods of sample transfer and during Nanodrop quantification) [2,4].

However, since in every problem there some means of solution, to combat these phenomena, physical separations of laboratory specimens are needed for reagent preparations and specimen's preparation. The use of ultraviolet light (UV) and chemical inactivation of surface contamination by sodium hypochlorite is recommended for the amplification room. Negative controls can also help in detection of foreign particles.

These artifact measurements can be minimized with the use of propidium monoazide (PM) prior to the nucleic acids extraction. Depending on the characteristic features of PM, it does not enter in cells that have structural integrity. They are normally connected to extracellular DNA or dead DNA (non-viable). PM intercalates into double stranded nucleic acids with chemical cross-links formed with bright light exposure [4,5].

The incorporation of uracil-N-glycosylase into the amplification products that predigest the reaction mixture helps to knockout some contamination [2]. Sometimes no PCR sample is detected (false negative results) in the presences of substances like; hemoglobin, triglycerides, heparinized blood or therapeutics drugs. This is caused by their inhibitory effects; even whereas the infection agent is presence but would not show up [2]. To troubleshoot these inhibitory properties, dilution of samples prior to amplification is recommended. At least the inhibitory substances will be inhibited while allowing the sample to be detected in the thermal cycler [2]. Internal positive control can easily be detected if inhibitory substances are present.

Above all, aseptic methods are strictly advised. The decontamination using DNA decontamination solution (DNAzap™) helps to prevent cross contamination, this kills any DNA left over. A No Template Control (NTC) can be run to clean the thermocycler prior to the test. The absence of no amplified product made it clear and safe for the sample to be run [11].

The Master Mix Selection and Reaction Design

The designing of the probe that are specific to distinct organism and the conditions in which these probes are optimized has been a great factor that determines the results of the real time PCR reaction. Out of no doubt, there are number of unidentified or non-discovered organism that brought difficulties in the sequence based-methods. This is due to the similarities or their proximity on the basis of phylogenetic. In fact among the commercial available kits and assay, there exist inconsistences of the assay platforms and this has gone as far as to different laboratories [5]. In addition, for Invasive Fungal diseases, the selection of specific primers often are accompanied with errors. This is due to the proximity of the genes of targeted organism [12].

The decision to choose among the commercial dyes often leads to opting for the cheaper wherein the non-specificities are most of the times tampered. While melting curve analysis is required for SYBR green amplified products, the TaqMan and molecular beacons gives an improved specificity [11,13,14].

The use of primer design software is strongly recommended. The melting temperature (Tm) of primer/probe is profoundly important to be noted in the amplification settings. In addition, the sequences of nucleotides and the inclusion of primers that can read exon-exon junction would knock down unwanted amplification organisms. Most often, variations might occur due to well-to-well however, this can be reduced by the use of ROX dye when added to the master mix [11].

Insufficiency of the target organism and impurity of nucleic acid

The use of Nanodrop to quantify the amount of DNA has a wide spread popularity. However, this method needs to be monitored as to how specific the machine can quantify nucleic acid. The purity of the DNA prior to the amplification expresses how accurate the results will be. If the sample size is too small to be amplified, or a foreign (non-targeted organism) is quantified by the Nanodrop machine, this will obviously give false negative results [5,15]. For RNA, once the quality is hindered, the quantitative results are mostly irrelevant. RNA must be pure and cleared away from DNA molecules and free of nucleases for extended storage. The efficiency of the RT reaction would be hugely limited if the RNA is impure. It is recommended that RNA should be extracted from either a fresh tissue or treated with RNA stabilization solutions [11].

The inability to target viable cells

Real time PCR as a highly sensitive device would detect any DNA (dead or alive) during the reaction so long it's the targeted DNA design for amplification. The life of cycle of diseases causing organism normally correlates with the stages of diseases development. Real time PCR still possesses some irregularities as to the identification of stages of diseases and as mentioned above, one of these is the inability to discriminate live cells from dead ones [12].

The costs involved in buying and maintaining

The establishment of fully fletch molecular laboratories for both research and clinical laboratories is still a dream that has not been realized. One of the reasons is the cost-effectiveness involved in buying equipment and reagents. Even after the establishment of the laboratory, with the most appropriate equipment, an extra

cost goes to the licensing and for the access to the database systems. Although with the use PCR that enables results of patients to be known in matter of house due to its accuracy in speed and the quickening of the Turn-Around-Time (TAT), the costs involved or attached per sample cannot match the cheaper or almost free of charge nature of culture-based methods. In fact the use of the most developed conventional methods like rapid antigen test results are available in matters of minutes [1,4,5,16].

Second to the cost involved in maintaining and buying of reagents are the limited available commercial kits. Until today, not all the pathogens have commercialized test kits to be detected by real time PCR. This insufficient number of test kits made the speedy device crawled with regards to its wide spreading. Until and unless, all the known pathogenic agents (bacterial, viral and fungal) have their highly specific test kits in commerce, the establishment of real time PCR in epidemiology and clinical laboratories will be hindered drastically [5].

Improper Storage and Specimen Processing

Real time PCR results are likely to be affected if proper and the very aseptic methods are not employed in the preparation and storing of samples and reagents prior to the usage. Collection time is very crucial as old stored nucleic acids for amplification in the refrigerator might become either denatured or cross contaminated by other specimens. The temperature range of particular samples as well, should be thoroughly understood. Not all DNA or RNA can perform optimal under the same temperature at the same period of storage. A typical example is *West Nile virus*; the viral titer is 10-fold lower per 24 hours at 28°C when compared to 4°C. DNA must be stored at 4°C or at -20°C and RNA ideally should be stored at -80°C while [2].

The sophisticated technical expertise

Among the modern techniques used in the modern laboratories, real time PCR still remains simple and convenient to use. However, the simpler the methods, the lesser the cautions are taken and the more the errors, are prone. Although assay designs are commercial and this comes with manufacturer instructions, the laboratory technicians decide to use traditional assays and this brought differentiations and either of those designs is not correct and result to incorrect results. Another cumbersome expertise that is crucial to any nucleic acid amplification is the extraction of DNA. DNA extractions come with different methods and these are based on one's own experiences without making thorough references to the standards specified by each group of organism. The resulting inappropriate method of extraction might not produce the targeted DNA instead an artifacts. The non-optimized reaction conditions and environments also affect the amplicon. Although not to a large extent, the specimen type and collection of the specimens also affect the outcome of the real time PCR efficiency. One of the unforeseen cases is the improper storage of both of the kits and known DNA molecules. DNA polymerase enzyme must only be stored in -21 degree until ready for usage [5].

One of the most cumbersome and rare understandings with regards to real time PCR are; the selection of the perfect chemistry, normalization gene and the methods of quantitation to be used. These technical confusions come as a result of the number of various choices available. Absolute versus relative quantitative methods are widely used when it comes to quantitation [17]. Also, in the aim of obtaining an accurate CT values, and for the most abundant sample, the baseline should be set at least two cycles earlier than Ct value [17].

The inability to detect an emerging new species

In research laboratories as in contrast to the clinical laboratories, the mere detection and quantification of targeted pathogen has never been a key interest. Research simply involves trying to discover the uncovered and this has to do with the flexibility of the method employed. Real time PCR, unlike culture-based methods does not have the ability to detect a new or emerging organism in a single reaction. The reaction conditions are design so much so that it is specific to only the targeted organism. Culture based-methods enable the growing and detection of new emerging organisms. This gives an upper hand to culture based methods. In a situation where PCR kits are to be commercialized to detect any emerging new species or genus; the time involved, the expertise and the cost would be enough to compelled one to resort to the traditionally culture based methods. Except broad-based PCR, none of the PCR type's technology can detect the occurrences of an organism whose primer is not assayed [4,5,12].

Issues regarding Multiplex PCR

In the business world, maximizing profit with the limited or minimized input is the main aim for any entrepreneur. So do the real time PCR world and disease diagnostic sectors. The amplification of multiple pathogens or pathogenic agent in a single reaction tubes with specific probes and proper primer design sounds more than science. Multiplex real time PCR has the ability to detect many DNAs of known organisms in single reaction tube with the optimized primer concentration, PCR buffers, DNA polymerase and deoxynucleosidetriphosphates (dent's) [18]. This is mostly used in diseases control units and as internal controls [4]. However, this reaction suffered a considerable sensitivity issues. Since one single reaction tube is to produce a result that is distinct to the targeted sequences, a single imbalance of the reaction mixture will produce nonspecific PCR products. In contrast to its singleplex counterpart, a promising specific product is gained.

In addition to non-specificity; the imbalance of the qualities of the DNAs of the targeted organisms to be co-amplified and a poor quality DNA or even less starting copy number will give rise to late threshold cycle. Although in the diagnostics laboratories, Multiplex PCR are known for detecting internal controls, optimizing multiplex real time PCR for detection of infectious organism is very cumbersome and alters to results while discriminating the sensitivity of the test.

The absences of quality control

The use of 'gold method' (reference method) in establishing concordant results has been the key to all the diagnostic laboratories. There are number of nucleic acid database established and open to public not only for PCR but also for other molecular methods. The variations that results from sample to sample are ought to be corrected by normalization. These methods are chiefly employed in gene expressions. Control genes are usually normalized against the PCR results and a typical example are 3-actin and housekeeping genes for quantitative PCR and in quantitative RT-PCR gene control respectively [17,19,20]. In addition, the addition of 18S rRNA is less variant in gene expression when compared with ß-actin or GAPDH as internal controls [19].

The absences of Proofreading

Disincorporation (misincorporation) is the term related to nucleotide incorporation errors in PCR methods. Lack of proofreading may often results to the errors in Nucleotides Corporation. The initial taq has not 3' to 5' exonuclease activity. Based on the previous assumptions, one of the reasons for this error is starting with the low target numbers. Failure to add the proofreading enzyme (Pfu, isolated from *Pyrococcus furiosus*) leads to mismatch in the any subsequent rounds. In addition to Pfu enzyme, there are commercial available enzymes that help knock down the mismatches and ensure efficient proofreading service (Efficiency in amplification). 5' exonuclease activity is used as an option despite its impacts in reducing the efficiency of reaction. In multiplex reactions, instead of this exonuclease activity, Stoffel fragment of Taq is added to inhibit the existence of exonuclease activity [4,20].

Detection of Antimicrobial Resistance

The use of real time PCR to detect antimicrobial resistance is thought to be a very reliable and faultless. However, the mechanisms of these resistance microorganisms comes in various strategies; one examples are while some are single genetic polymorphisms, many others are in multiple genetic polymorphisms. A very effective assay is required to detect these widely varied sequences for the antimicrobial to be detected. To worsen the situation, until today many types of molecular genetics mechanisms for some antimicrobial resistances are not yet known. Another challenging factor is the evolving nature of the antimicrobial resistances. A constant development of new assays are required in order to keep tract of the detection accuracy, otherwise using wrong assay for a particular resistance detection would be the order of the day. In clinical laboratories, the waiting time for these would experiences delays when the use of fastidious organisms or very slow growing organisms that will take days before they would be characterized by their susceptibility profiles [4].

Conclusion

It is believed that science is always the first when it comes to life. However, there has been a micro politics in every sector of our lives recently. The challenges aforementioned will be a thing of a past in a very near future. As new inventions are coming on our ways, even more improved technologies would create better outputs.

Despite these inconsistences, PCR as of do doubt has offered the scientific world ranging from reduced time, reduced space requirements and risk of contamination. Both in patients and outpatients settings, an appropriate therapy are ensured within hours with the use of PCR machines. The data analysis system brought by quantitative real time PCR is unbiased. It is one of the most common methods for measuring gene expression.

The inculcation of internal positive controls (IPC) with a well-designed multiplex primers and probes serves as a better quality control.

Against all odds, a well-designed real time PCR experiment will still produce the most sensitive, efficient, reproducible and the fastest results in our modern day laboratories.

Bibliography

1. Bajinka O and Secka O. "Integration of Molecular Methods into Microbiological Diagnostics". *Applied Micro Open Access* 3 (2017): 130.

2. Morshed GM. "Molecular methods used in clinical laboratory: prospects and pitfalls". *FEMS Immunology and Medical Microbiology* 49.2 (2007): 184-191.

3. Bajinka O and Ozdemir G. "The Validity of Singleplex and Multiplex real time PCR Detection and Quantification of Waterborne Pathogens from Domestic to Industrial Water". *Research Journal of Pharmaceutical, Biological and Chemical Sciences* 3.1 (2017): 25.

4. Persing HD., *et al*. "Molecular microbiology: diagnostic principles and practice Book 2nd edition" (2003).

5. David J Speers. "Clinical Applications of Molecular Biology for Infectious Diseases". *Clinical Biochemist Reviews* 27.1 (2006): 39-51.

6. Gordanpour A., *et al*. "MicroRNA Detection in Prostate Tumors by Quantitative Real-time PCR (qPCR)". *Journal of Visualized Experiments* 63 (2012): e3874.

7. Nikitina TV., *et al*. "Use of the real-time RT-PCR method for investigation of small stable RNA expression level in human epidermoid carcinoma cells A431". *Tsitologiia* 45.4 (2003): 392-402.

8. Girones R., *et al*. "Molecular detection of pathogens in water--the pros and cons of molecular techniques". *Water Research* 44.15 (2010): 4325-4339.

9. Francino O., *et al*. "Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis". *Veterinary Parasitology* 137.3-4 (2006): 214-221.

10. Antinori S., *et al*. "Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature". *Clinical Infectious Diseases* 44.12 (2007): 1602-1610.

11. Stephen A Bustin and Tania Nolan. "Pitfalls of Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction". *Journal of Biomolecular Techniques* 15.3 (2004): 155-166.

12. AM Sousa and MO Pereira. "A prospect of current microbial diagnosis methods". Microbial pathogens and strategies for combating them: science, technology and education (A. Méndez-Vilas, Ed) (2013): 1429-1438.

13. Yin JL., *et al*. "Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I". *Immunology and Cell Biology* 79.3 (2001): 213-221.

14. Mortarino M., *et al*. "Quantitative PCR in the diagnosis of Leishmania". *Parassitologia* 46.1-2 (2004): 163-167.

15. S Broeders., *et al*. "Guidelines for validation of quantitative PCR methods". *Trends in Food Science and Technology* 37.3 (2014): 115-126.

16. Barbara A Brown-Elliott., *et al*. "Clinical and Laboratory Features of the Nocardia spp. Based on Current Molecular Taxonomy". *Clinical Microbiology Reviews* 19.2 (2006): 259-282.

17. Marisa L Wong and Juan F Medrano. "Real-time PCR for mRNA quantitation". BioTechniques 39.1 (2005): 75-85.

18. MJ Espy., *et al*. "Real-Time PCR in Clinical Microbiology: Applications for Routine Laboratory Testing". *Clinical Microbiology Reviews* 19.1 (2006): 165-256.

19. Heid A C., *et al*. "Genome Methods Real Time Quantitative PCR". Genome Research 6.10 (1996): 986-994.

20. Bustin SA and Mueller R. "Real-time reverse transcription PCR and the detection of occult disease in colorectal cancer". *Molecular Aspects of Medicine* 27.2-3 (2006): 192-223.

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