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REVIEW ARTICLE

A review on challenges in the development of multiplex assay for quantitative estimation of viral pathogens

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Abstract

HBV, HCV, and HIV are the primary infectious agents that can be transmitted via exposure to body fluids and affect human health to various degrees. Controlling the spread of these viruses to uninfected individuals is a major task. These viruses have highly competent replication potential and are undetectable by serological markers at an early phase of infection. Molecular techniques on the other hand, by their enhanced sensitivity, selectivity, short detection time, absolute quantification with unparalleled precision have revolutionized the area of diagnosing multiple viral infections simultaneously. Furthermore, detecting multiple viral infections in a single assay is time saving and cost effective as compared to assays designed to detect an individual pathogen. Various digital and real-time PCR-based qualitative and quantitative assays have been developed for estimation of these viruses simultaneously and individually, and very few of them are licensed by the food and drug administration (FDA). Furthermore, the alleged potential of nanotechnology and digital droplet technology has attracted the attention of viral diagnostic research across the globe. In this review, we describe various commercially available technologies for identification and quantification of these viruses and the challenges involved in the development of a cost-effective multiplex assay for simultaneous detection of these viral pathogens.

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Introduction:-

Among the majority of infections reported so far by various pathogens, viruses are one of the major class of pathogens prominently affecting human health all over the world. Apart from mono infection, the incidence of co-infection with various viruses is an additional troublesome situation observed in the case of HIV infection. When an individual suffers from co-infection by various pathogens and causing an ambiguous illness leading to a phenomenon of syndrome then a rapid diagnosis of such multiple pathogens is an ultimate requirement of the clinician to provide prompt and appropriate therapy¹. Furthermore, drug-resistant viral strains escaping the drug pressure have been emerged and reported more than a decade ago². Determination of viral load (copies of nucleic acid) in peripheral blood is being used widely in the clinical management of viral infections and to study the efficacy of antiviral therapies for controlling the emergence of drug-resistant variants.³ A variety of molecular techniques has been developed for rapid diagnosis of infectious syndromes⁴. The most commonly used methods to quantify viral

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DNA or RNA include real-time (RT)-PCR, isothermal nucleic acid sequence-based amplification (NASBA), branched DNA assay, DNA/RNA/Protein microarray, next generation sequencing (NGS)⁵ and upcoming methodology like digital droplet PCR⁶. Apart from this, there are advances in micro fluidics, micro-electromechanical systems (MEMS), and nanotechnologies which will show promise for self-contained, minimally instrumented point-of-care diagnostic systems⁷. These recently developed technologies provide new insights into the early dynamics of viral replication during acute or persistent phases of viral infection. This review focuses on the availability of these technologies and the challenges involved in the development for quantification of single or multiple viral infections. A better knowledge of the multiple viral infections with their quantitative load will further improve the situation by virtue of its reduced cost, reliable results, conservation of precious samples and increased throughput.

Replacing age-old Diagnostic Technologies with advanced platforms:-

Electron microscopy was seldom used as a diagnostic technique since 1938 when first electron micrograph of poxvirus was published to get sufficient resolution of viral particle to confirm its infection⁸. Furthermore, cell culture based technique was used for confirmation of the pathogen. However, serological tests identifying the formation of antigen-antibody complex, namely enzyme-linked immunosorbent assay (ELISA), rapid and western blot were developed along with in-vitro tissue culture albeit it was considered as a gold standard method a few years ago. Due to delay in identification of the viral infection from an early treatment point of view, the cost involved and the lack of sensitivity and specificity, it was essential to develop rapid molecular tests for identification and quantification of viral infection. Recently, rapid and sensitive molecular techniques are developed for the quantitation of a variety of infectious viruses based on the detection of the fluorophore-labeled oligonucleotide which appropriately detects multiple targets in the same PCR reaction (multiplex PCR)⁹. Earlier methods like classical PCR could not fulfill all of the criteria during quantification and thus was replaced by Real-Time chemistries to detect and quantify PCR amplicon during the early phases of the reaction and provide a distinct advantage over traditional PCR. Traditional methods used agarose gels where detection of the amplicon was based on size discrimination and it was the end-point of the PCR reaction. The final outcome of the reaction was not very precise and was likely to vary from sample to sample and is time-consuming. Furthermore, agarose Gel resolution is very poor, about 10 fold while Real-Time PCR can detect as little as a two-fold change with minor variabilities in the yield¹⁰.

Current platform of Real-time multiplex PCR can detect, differentiate, and provide a quantitative result for many different gene targets without influencing the detection of one target gene cross-talking with the others and without loss of sensitivity⁹. Furthermore, Real-Time PCR methods are not prone to carryover contamination and sensitive enough to cover broad dynamic range of detection/quantification on the order of 1.60 to 7.0 log₁₀ copies/ml i.e. 40 - 1 x 10⁷ million copies/ml for HIV-1 RNA using Abbott Real-Time PCR¹¹ while for HBV DNA linear detection showed over 7 logs for genotypes A to D using CobasTaqMan assay¹² and it was 10 to 15 international units (IU)/ml for HCV RNA using COBAS Ampliprep assay¹³. The fluorescence-based quantitative real-time PCR (qPCR) with its capacity to detect and measure minute amounts of nucleic acids in a wide range of samples from numerous sources, is the enabling technology at par excellence for molecular diagnostics, life sciences, agriculture, and medicine^{14,15}. The practical and theoretical simplicity of real-time PCR, together with its combination of speed, sensitivity, and specificity in a standardized assay, has made it the touchstone technology for nucleic acid quantification. In addition to its use as a diagnostic tool, many research-based applications have been developed. These include microbial quantification, gene dosage determination, and identification of trans-genes in genetically modified foods, risk assessment of cancer recurrence, and applications for forensic use¹⁶⁻¹⁸.

Application and interpretation of results in diagnostics:-

For interpretation of results, it is essential to provide sufficient experimental details to critically evaluate the quality of the results presented or details of the repeated experiments, precisely about the information of sample attainment and handling, RNA quality and integrity, reverse-transcription details, PCR efficiencies, and all the analysis parameters. The information provided along with qPCR assays must include database accession numbers of each target and reference gene, the exon locations of each primer and any probe, the sequences, and concentrations of each oligonucleotide, including the identities, positions, and linkages of any dyes and/or modified bases. For reproducibility of the experimental data or all the assay parameters viz the concentration and identity of the polymerase, the amount of template (DNA or cDNA) in each reaction, the Mg ion concentration, the exact chemical compositions of the buffer (salts, pH, additives), and the reaction volume are also important. It is also essential to mention the details of the instrument used and the PCR cycling conditions. Usually, the consumables used can affect

thermal cycling parameters. The degree of transparency of the plasticware used during experiments is also important because different plastics exhibit substantial differences in fluorescence reflection and sensitivity¹⁹. As PCR efficiency is highly dependent on the primers used, their sequences must be thoroughly worked out. Additionally, in silico tools such as BLAST or equivalent specificity searches are useful for assay design. Specificity must be validated with direct experimental evidence like gel electrophoresis, melting profile, DNA sequencing, amplicon size, and/or restriction enzyme digestion. A marked presence of primer dimers produces a lower PCR efficiency in probe-based assays and may generate false positives in assays based on DNA binding dyes like SYBRGreen I. The details of primer optimization in the form of annealing temperature, Ct values, plots of fluorescence Vs cycle number, and/or melt curves must be presented after data analysis²⁰. The assay performance characteristics viz PCR efficiency, linear dynamic range, limit of detection (LOD), and precision must be determined. PCR efficiency is particularly important when reporting mRNA concentrations for target genes relative to those of reference genes. PCR amplification efficiency must be established by means of calibration curves because such calibration provides a simple, rapid, and reproducible indication of the mean PCR efficiency, the analytical sensitivity, and the robustness of the assay. Differences in PCR efficiency will produce calibration curves with different slopes^{21, 22}. The dynamic range is established from a linear reaction of a calibration curve with the highest to the lowest quantifiable copy number. Depending on the template used for generating calibration curves, the dynamic range should cover at least 3 orders of magnitude and ideally, should extend to 5 or 6 log₁₀ concentrations with the LOD at which 95% of the positive samples are detected.

Pros and cons of qPCR/ real-time PCR:-

Recent literature substantiates variation in qPCR results which includes temperature differences affecting the completion of annealing and/or denaturation, concentration differences introduced by pipetting errors, and stochastic variation. Standard deviation is a measure of precision that typically varies in qPCR with concentration and with decreasing copy number²³. Ideally, intra-assay variation (repeatability) should be displayed in figures as SD error bars or as CIs (confidence intervals) on calibration curves with replicate samples. For diagnostic assays, it may also be necessary to report inter-assay precision (reproducibility) between sites and different operators. Recently, it is reported that the ability to multiplex greatly expands the power of qPCR analysis²⁴, particularly when applied to the simultaneous detection of point mutations or polymorphisms²⁵. Multiplexing requires the presentation of evidence demonstrating that accurate quantification of multiple targets in a single tube without any impairment i.e., assay efficiency and the LOD are the same as when the assays are run in uniplex fashion. This concern is of particular importance when targets of appreciably lower abundance are co-amplified with highly abundant targets. Additionally, there are various other advantages of performing a multiplex PCR. False negatives are often revealed in multiplex assays because each amplicon provides an internal control for the other amplified fragments. The expense of reagents and preparation time is less in multiplex PCR than in systems where several tubes of uniplex PCRs are used. Thus a multiplex reaction is ideal for conserving costly reagents and precious samples. The exponential amplification and internal standards of multiplex PCR can be used to assess the amount of a particular template in a sample. To quantify templates accurately by multiplex PCR, the amount of reference template, the number of reaction cycles, and the minimum inhibition of the theoretical doubling of product for each cycle must be accounted.

Global and Indian Scenario of HIV, HBV and HCV:-

Human immunodeficiency viruses (HIV-1 & HIV-2), Hepatitis B virus (HBV) and Hepatitis C virus (HCV) are the most common chronic viral infections all over the world as they share similar transmission routes including sexual contact, blood-blood contact, and injecting drug usage^{26, 27}. These viruses cause fatal, chronic and life-threatening disorders. The prevalence of these viruses varies by geographical locations and population. HIV/AIDS remain an important infectious disease and public health concern worldwide. There are 35 million people living with HIV and 13.6 million people had access to antiretroviral therapy as reported in 2014^{28, 29}. Infection caused by Hepatitis B virus is a major global health problem leading to potentially life-threatening liver infection and. It can cause chronic liver disease and chronic infection and puts people at high risk of death from cirrhosis of the liver and liver cancer. More than 240 million people have chronic (long-term) liver infections. More than 7, 80,000 people die every year due to the acute or chronic consequences of hepatitis B^{30, 31}. Hepatitis C is a major blood-borne infection of public health importance and is mainly associated with the development of liver cirrhosis, hepatocellular carcinoma, liver failure, and death³². Hepatitis C virus (HCV) is a globally prevalent pathogen causing >185 million infections worldwide and is a leading cause of death and morbidity especially in HIV-positive patients on highly active antiretroviral therapy³³⁻³⁵.

Co-infection of HIV with HBV and/or HCV is very common in certain populations, such as intravenous drug users (IVDUs) who often share the contaminated needles/syringes for intravenous drug injection. It has been reported that the prevalence of HIV-HCV co-infection among IVDUs can surpass 90% in certain populations all over the world³⁶.³⁷ while in the same group, the rates of HIV-HBV co-infection are reported as high as 10–20%³⁸. It has been reported that HBV/HIV co-infection leads to increased morbidity and mortality as compared to HIV or HBV or HCV mono-infections³⁸. In view of the high replication rates and undetectable immunological and serological markers, early detection of these viruses upon transmission is crucial. Various nucleic acid assays have been developed for diagnostics and therapeutic monitoring of these infections. In the past two decades, rapid and sensitive molecular techniques such as PCR and Real-time PCR have revolutionized the detection of a variety of infectious viruses, including HIV, HCV, and HBV³⁹⁻⁴².

Importance of quantitative estimation of viruses:-

Improvement in the molecular technologies have given better methodologies to perform estimation of any viral infections rapidly over traditional approaches wherein multiplexing for simultaneous detection of many pathogens is possible in a single assay. This approach is the optimal use of the technology that will not only be responsible for lowering down the cost and time but also will be useful in using less volume of sample for rapid diagnosis to start with appropriate drug therapy at earliest. Thus, various PCR-based assays have been developed for detection and discovery of multiple pathogens simultaneously⁴³⁻⁴⁶. The microarray is one of the useful tools used in the identification of viruses from single known source of origin however it is very difficult, laborious and less sensitive for detection of various viral targets as compared to real-time PCR. Apart from this, the data obtained from microarray analysis needs to be further confirmed using real-time PCR. Additionally, digital droplet PCR technology has been developed for quantification, however; it is again restricted to quantify two viruses simultaneously. Furthermore, accurate quantification of lower concentrations can be obtained via digital droplet PCR which follows a principle of Poisson distribution⁴⁷.

Estimation of Human immunodeficiency virus (HIV-1) plasma RNA viral load and CD4 counts have become an important crucial step for understanding HIV pathogenesis and providing pre and post exposure care to the HIV-infected individuals⁴⁸⁻⁵⁰. Along with this the accuracy, precision and %CV in the measurement of such analytical parameters in HIV-1 RNA viral copies and CD4 T lymphocyte are the keystones of modern HIV care^{51, 52}. Estimation of HIV viral load levels is the analytical measure of individual's infectivity and their risk for progression towards AIDS^{53, 54}. The significant suppression of plasma viremia quantified using FDA approved viral estimation assay in consecutive visits is the indication of successful antiretroviral therapy^{51, 55-57}. It is mandatory for the manufacturers of the kit to provide an assay which will be accurate, reproducible, and cost-effective and at the same time feasible for the diagnosis of emerging subtypes, circulating recombinant forms (CRFs) and unique recombinant forms (URFs)⁵⁸⁻⁶⁰. Co-existence of different viral genotypes within a given population also increases the risk of inter-genotype recombination, and the emergence of recombinant forms establishing new variants should be predictable⁶¹. Such new circulating recombinant forms should be incorporated into the global dimension of the viral epidemics and carefully monitored as their prevalence may increase at the expense of the parental strains as previously described for HIV-1 B/F recombinants⁶². As compared to HIV-1, the HIV-2 virus is less pathogenic and also has a lower plasma viral load. This becomes the challenge for the development of viral load monitoring assay in HIV-2 positive patients. Presently there is the commercial availability of HIV-1 viral load estimation kits which are not targeted to detect any of the subtypes of HIV-2 sequence but attempt have been made to develop a kit for HIV-2 viral load estimation focusing only HIV-2 subtype A and subtype B⁶³⁻⁶⁶. A study conducted on quality control comparison of HIV-2 viral load quantification between nine laboratories have highlighted the variation in HIV-2 viral load determination and indicated the lack of a standardized assay⁶⁷.

Current technologies to quantify HIV, HBV and HCV:-

Quantitative estimation of HIV RNA:-

Currently there are various commercially available FDA-approved assays available for quantitative estimation of HIV-1 including Real-Time HIV-1: *m2000rt* instrument (*m2000rt*; Abbott Molecular Diagnostics), COBAS AmpliPrepTaqMan HIV-1 48 (CAP-CTM; Roche Molecular Diagnostics), EasyQ HIV-1 v1.2 assay (EQ; bioMérieux), and VERSANT 3.0 HIV-1: VERSANT 440 instrument (bDNA; Siemens). The first three assays are using the quantitative approach in real-time PCR to estimate HIV RNA copies present from plasma samples while fourth assay namely VERSANT is a branched DNA endpoint detection assay⁶⁸. At present, more commonly used assay is the *m2000rt* assay which targets a conserved region of the *pol-integrase* genes and amplifies HIV-1 RNA using a partially double-stranded qrtPCR method. In this assay, a partial sequence of the pumpkin polymerase gene

acts as the internal control. According to the literature provided by the manufacturers, this assay reliably detects HIV-1 group M, A–D and F–H viruses, several CRFs, including CRF01-AE and CRF02-AG as well as group N, and O. The purified RNA is extracted from a minimum of 0.6 ml plasma sample using the *m2000sp* automated extractor, and quantitative rtPCR amplification and detection is done using the fully automated *m2000rt* instrument. The dynamic range of the assay is 1.60 to 7.0 log₁₀ copies/ml i.e. 40-1 x 10⁷ million copies/ml.

Quantitative estimation of HBV DNA:-

As hepatitis B is a serious and common infectious disease of the liver and affecting millions of people throughout the world and its early diagnosis is obligatory for early recovery⁶⁹⁻⁷¹. Initially, quantitative estimation of HBV used to be tested by real-time PCR using commercial SYBR-Green reaction mix (Qiagen, Hilden, Germany) and primers specific to the S gene with primers spanning to amplify a 98 base pair product from positions 379 to 476 of the HBV genome. HBV-DNA concentration was usually calculated from a four-point standard curve [1.5 x 10⁸, 1.5 x 10⁶, 1.5 x 10⁴ and 1.5 x 10²copies/ml] and calibration of this standard curve were confirmed by comparison with an international HBV-DNA standard (97/746; NIBSC, Potters Bar, UK). The detection limit of the RT-PCR assay was 260 DNA copies or 2.41 log₁₀ copies per ml⁷², although the qualitative limit of detection for the assay was established as 200 DNA copies or 2.3 copies per ml. Though singleplex quantitation of HBV used to be done initially at sporadic places, the HBV DNA testing was globally implemented only after 2004 when multiplexed commercial NAT assays that included simultaneous detection of HBV, HCV and HIV-1 nucleic acids became available but the cost of this assay was high and beyond the reach of a common man⁷³.

Quantitative estimation of HCV RNA:-

At the end of 19th century, Blood banks around the world established first mini-pool NAT for detection of HCV rapidly followed by duplex testing of HIV-1 on a routine basis but subsequently many amplification methods such as transcription-mediated amplification/nucleic acid sequence based amplification (TMA/NASBA), ligase chain reaction (LCR), and branched DNA signal amplification assay (bDNA assay) were developed which are useful for identification of multiple pathogens simultaneously^{74, 75}. Although the feasibility of these applications has been demonstrated by various commercially available platforms, many testing centers are finding it difficult to implement NATs due to the complexity of target selection, low throughput, and inadequate sensitivity and specificity.

Discussion:-

Usually, in duplex real-time PCR system, the TaqMan chemistry is used to measure PCR product accumulation through a dual-labeled fluorogenic probe (TaqMan probe) and the fluorescent signal is generated by means of 5'-nuclease activity that separates a fluorescent reporter dye and quencher dye⁷⁶⁻⁷⁷. Amplifying and detecting HBV DNA, HCV and HIV-1 RNA with equal efficiency in the multiplex assay have been found to be challenging. Since both HIV-1 and HCV amplifications involve reverse transcription (RT) step, RT primer concentrations and thermal cycling conditions needs to be adjusted properly so that the amplification efficiencies for each of the types of amplicons of viral targets along with internal control will be approximately equal during the exponential phase of the reaction⁷⁸. The number of fluorogenic detection probes should be conserved among most viral genotypes and subtypes so that they will be hybridized to target sequences within their respective target amplicons in amplification step. Primer-template hybrids will be stabilized when the thermal-stable enzyme extends the primer in the polymerization step. As the fluorogenic probes can't be extended, the detection probes should be designed to be longer than the primers to achieve a stable probe-template hybrid^{79, 80}.

Furthermore, in multiplex real-time PCR where quantitation of HIV-1, HBV and HCV is desired then there is a need to include three detection probes for HIV-1, HBV and HCV which could be labeled with the three different fluorogenic reporter and quencher dyes so that the fluorescent signals generated from all three targets have a different wavelength. Along with this the internal control also should be labeled with a different fluorogenic reporter dye and the same quencher dye as that of the target. Target and internal control probes will generate fluorescent spectrum contributed by individual component dye spectra which need to be analyzed by the system specific analyzer to evaluate available copy number of the viral target⁸⁰.

Considering the genetic diversity of the viral pathogens and their recombination, it is very difficult to identify the particular pathogen precisely and this limits the confirmation of pathogen by any molecular assays. The targets of the newly developed assays need to be reassessed as viral mutations and their variability continues to grow. An ideal target for a viral load assay would be conserved across all types of the pathogens. Genetic variation in viral pathogens or extreme divergence within their subtypes may significantly affect the ability to detect and quantify the

viral RNA in clinical specimens. Non-detection, under-quantification, or even over-quantification of plasma viremia, has the potential to cause serious errors in patient care. Hence, close collaboration between clinicians and laboratory specialists is essential to recognize challenges of ongoing viral diversity and their identification. Establishing a laboratory network that has the capacity to have samples with unusual characteristics tested by an alternative assay, and in the case of discordant results send the sample for further sequencing characterization, is an important step in building the necessary infrastructure to monitor local and global viral diversity hence forth⁸¹.

An approach of validated multiplex real-time PCR may need to be emphasized in every set up for rapid identification of multiple pathogens and also close monitoring of genetic diversity of viruses leading to the effective application of antiviral therapy for better clinical management of infected individuals. A qualitative assay would, therefore, be used for monitoring until such time as virus again becomes detectable on therapy, indicative of viral relapse. This approach has been successfully used to treat many viral infections and may be effective and much less expensive for monitoring HIV-1 infected individuals even when highly active antiretroviral therapy (HAART) has effectively suppressed HIV virus replication in plasma below detectable levels. A multiplex assay with high-throughput and high-sensitivity are needed to meet the testing requirements of multiple pathogens simultaneously for samples collected at various testing centers⁸².

Conclusion:-

Identification of emerging viruses and their variants remains a major driving force for the development of technically innovative and challenging new molecular technologies for diagnosis of such viruses. This challenge though fulfilled in the developed world but still has a concern in the developing world where the major burden of HIV, HBV, HCV and viral diversity exists. Molecular virology methods have been increasingly introduced focusing mainly on either uniplex or multiplex identification and quantification of nucleic acid to reduce the risk of co-infecting viral pathogens. However, the challenges concerning sensitivity, specificity, user-friendly, time and cost effectiveness are still in the pipeline for development of multiplex assay. Further characterization of such confirmed viral pathogens may shed light on genetic diversity and drug resistance pattern to help improve strategy of drug combinations. Although there are many technical and financial challenges intrinsic to wide-scale deployment of HIV-1 viral load assays in resource-limited areas, our goal should be justifiable for simultaneous identification and quantification of multiple viral targets collectively.

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