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Review Article

Biotechnology Section

Automated Triplex (HBV, HCV and HIV) NAT Assay Systems for Blood Screening in India

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ABSTRACT

This review is confined to triplex nucleic acid testing (NAT) assays to be used on fully automated platform. Around the world, these assays are being used at various transfusion medicine centres or blood banks to screen blood units for HBV, HCV and HIV. These assay systems can screen up to 1000 blood units for HBV, HCV and HIV simultaneously in a day. This area has been dominated by mainly two manufacturers: M/s Gen-Probe-Novartis and M/s Roche Molecular Systems. The triplex NAT assay systems of both manufacturers are licensed by United States Food and Drug Administration. There is not much awareness about the technology and procedures used in these assays. The main objective of this review is to create awareness about the technology and procedure of these assays.

Keywords: Individual donations, Molecular diagnostics, Transfusion transmitted infections

INTRODUCTION

Each country should prepare a blood policy as a part of the national health policy, to make available safe blood for the transfusion needs of its population. A significant number of donated blood units remains unsafe as it is not screened with advanced technology or not screened for all the major transfusion transmitted infections (TTIs). The implementation of efficient and effective blood screening programmes have reduced the risk of TTIs dramatically over the last two decades in various countries [1,2]. As per Drug and Cosmetics Act, 1940 and the rules there in, the 3rd generation serological assays are mandatory for blood donor screening for HBV, HCV and HIV in India [3]. The donations screened by such assays are still at risk for HBV, HCV and HIV infections. The prevalence of HBV, HCV and HIV is high in India in comparison to western countries where this technology has been implemented for blood screening. In the developed countries NAT was started in the late 1990s and early 2000s and presently around 33 countries in the world have implemented NAT for HIV and 27 countries for HBV [4]. NAT technique is highly sensitive and specific for viral nucleic acids. It is based on amplification of specific sequences of viral RNA or DNA and detects them earlier than the serological screening methods thus, shortens the window period of HBV, HCV and HIV infections. NAT also adds the benefit of resolving false reactive results of serological methods, which is very important for donor notification and counselling [5]. Realizing this fact there is an urgent need for higher sensitive assay like NAT to add an additional layer of safety to the blood for transfusion. In India, some transfusion medicine centres including government and private have started blood screening for HBV, HCV and HIV by NAT. NAT has been shown to reduce window periodof HBV to 10.34 days, HCV to 1.34 days and HIV to 2.93 days [6]. NAT assays can either be performed on individual donations (ID) or on mini-pools (MP) to screen the blood for transfusion transmitted infectious agents. In addition to NAT assays which target individual viral nucleic acids, multiplex or triplex NAT screening assays have been developed which can detect DNA or RNA from multiple infectious agents simultaneously. The major hindrance in implementing NAT for blood screening in India is its high cost. In order to make it cost-effective the method of pooling different donations is being followed by various institutions. NAT was introduced in Germany in 1997, and from 1997 to 2005, around 31 million blood donations were screened by minipool NAT, with pool sizes of 96 donations. During this screening 23 HCV, 2 HIV-1 and 43 HBV NAT yield were detected [7]. Different countries and institutions are adopting different mini-pool (MP) sizes viz. 24, 16, 8 and 6 donations. A study from India also suggested NAT testing in different pool sizes to make it cost-effective [8]. There are some disadvantages of MP-NAT: (a) viral concentration gets diluted which may lead to decrease in sensitivity at 100% confidence interval e.g. if a donation with 30 copies/ml of HIV-1 is pooled with 5 other donations and these 5 donations are negative for HIV-1, then the final concentration of HIV-1 becomes 5 copies/ml. Therefore, there are chance for a NAT assay to miss this infection if the lower limit of detection (LOD) is less than 5 copies/ml at 100% confidence interval; (b) If the minipool is found reactive, all pooled donations needs to be tested individually to identify the reactive unit(s); (c) All pooled donations are delayed until the individual resolution NAT testing is complete. Therefore, to overcome these limitations individual donor NAT (ID-NAT) testing is being suggested and is available from the manufacturers of NAT assays. In India, M/s Roche has mainly promoted NAT testing in minipool of 6 individual donations (MP6), while M/s Gen-Probe-Novartis is promoting NAT testing for both individual donation and in minipool of 16 individual donations (MP16). The ID-NAT has been observed more sensitive by different groups when compared to MP-NAT with pools of 16 or 8 or 4 samples [9,10].

QUALITY EVALUATION OF NAT ASSAYS

Most countries have at least one well-established laboratory with the relevant expertise and experience that could be designated as a reference laboratory. Our institute is a testing laboratory for Central Drugs Standard Control Organization (CDSCO), New Delhi, for quality evaluation of NAT assays intended to be used for blood donor screening, infection diagnosis and viral load monitoring. Other institutes such as AIIMS, New Delhi, SGPGI, Lucknow and NABL accredited laboratories also test these assays when recommended by CDSCO. We have developed well characterized plasma panels for evaluation of NAT assays. The triplex blood donor screening assays are evaluated with HBV, HCV, HIV positive and negative plasma panels for sensitivity and specificity parameters. The laboratory has also acquired WHO international standards for HBV, HCV and HIV-1&2. These standards are used for internal quality checks at an interval of one year. The laboratory also participates in HCV NAT proficiency testing scheme organised by European Directorate for the Quality of Medicines and Healthcare.

AUTOMATED TRIPLEX NAT ASSAYS

In India and around the world, automated triplex NAT assays for screening blood for HBV, HCV and HIV are mainly available from two manufacturers M/s Gen-Probe-Novartis (GPN) and M/s Roche Molecular Systems (RMS) [11]. GPN offers three triplex NAT assays, ProcleixUltrio, ProcleixUltrio Plus and ProcleixUltrio Elite assays. These assays are used in conjunction with their fully automated platforms, Procleix Tigris system and Procleix Panther system [12-14]. On the other hand RMS offers cobas Taq Screen MPX and cobasTaqScreen MPX v2.0 assays. These MPX assays are to be used on their fully automated platform cobas s201 system. Recently, in 2014, RMS has launched a new fully automated high through put platform cobas 6800 [15-18].

Technology Principles

ProcleixUltrio, ProcleixUltrio Plus and ProcleixUltrio Elite assays of GPN are based on Transcription Mediated Amplification (TMA) whereas, cobasTaqScreen MPX and cobasTaqScreen MPX v2.0 assays of RMS are based on PCR/RT-PCR technology [12-16].

TMA technology for Procleix assays was developed by Hologic, a GPN partner. This has been patented by them. The *invitro* amplification of viral nucleic acid is this technology is similar to *invivo* replication of HIV nucleic acid. The procedure of TMA based assay can be described in three steps: Target capture, Amplification and Detection.

Target capture: In this step the samples are lysed to release viral nucleic acid and viral genomic sequence specific complementary capture probes hybridise to the target sequences. The hybridised nucleic acids are then captured onto magnetic micro particles that are separated exploiting magnetic field and unbound or non-specific material/ nucleic acid is washed out to minimize potential inhibitors.

Amplification: Amplification of hybridized nucleic acid is carried out with two enzymes MMLV reverse transcriptase and T7 RNA polymerase. The reverse transcriptase generates cDNA containing promoter sequences for T7 RNA polymerase from the target sequence. RNA polymerase produces RNA amplicons from cDNA template through the process of transcription. Some of the RNA amplicons reenter the TMA process and serve as template for new rounds of amplification. It is claimed that billions of copies are generated in less than one hour.

Detection: Detection is achieved by hybridization protection assay (HPA). In HPA, sequence-specific single stranded nucleic acid probes labelled with acridinium ester (AE) hybridise to the RNA amplicons generated through TMA. The selection reagent inactivates AE label on unhybridised probes. Therefore, background signal is minimized and at the same time hybridised probes get differentiated from unhybridised probes. The longer-lasting chemiluminescent signal generated by the hybridised probe is detected by a luninometer and reported in terms of relative light units (RLU). The number of RNA amplicons generated is directly proportional to the number of target nucleic acid molecules in the starting sample. Only one molecule of AE-labelled probe hybridises to a RNA amplicon. The RLUs obtained is therefore a measure of initial concentration [12-14].

CobasTaqScreen MPX and CobasTaqScreen MPX v2.0 are based on reverse transcription and PCR amplification. These assays also involve three main steps: specimen preparation, amplification and detection.

Specimen preparation: Lysis reagent carries out viral lysis leading to release of nucleic acids. Due to net negative charge of nucleic acid in presence of lysis reagent, the nucleic acids bind to added magnetic particles and unbound material is washed out. Purified nucleic acids are eluted.

Amplification: Reverse transcription and amplification are carried out by a recombinant enzyme Z05 DNA polymerase in single step. Z05 DNA polymerase has reverse transcriptase and DNA polymerase activities in presence of manganese. Reverse transcriptase activity generates cDNA in case of HCV and HIV. After reverse transcription amplification of cDNA/DNA is carried out, which is similar for HBV, HCV and HIV. During PCR amplification, the thermal cycling denatures the target amplicon to single stranded DNA and DNA polymerase converts these single strands into double-stranded DNA amplicons. This process is repeated for multiple cycles, with each cycle number of DNA amplicons get doubled.

Detection: Detection occurs simultaneously with amplification and sequence-specific dual labelled probes are used to detect the presence of the target. These probes are labelled with reporter dye at 5'end and quencher dye at 3'end. The reporter dye fluorescence is supressed by the quencher dye. This quenching effect continues till the both dyes are in close proximity and is known as Forster resonance energy transfer quenching mechanism. During amplification, the probes hybridize to target complementary DNA sequences and are cleaved by the 5' to 3' exonuclease activity of Z05 DNA polymerase at the time of primer extension. This leads to breakage of the close proximity of these dyes and fluorescence occurs. With each PCR cycle a number of probes are cleaved and therefore with each cycle fluorescence signal increases. This fluorescence is detected by detecting device and reported [15].

TECHNOLOGY: PROCLEIX ASSAYS VS COBAS TAQSCREEN MPX ASSAYS

In Procleix assays the selection of specific targets and probe binding occurs during specimen preparation or nucleic acid extraction while in cobasTaqScreen MPX assays it occurs during amplification. TMA in Procleix assays are carried out by two enzymes reverse transcriptase and RNA polymerase but RT-PCR/ PCR in cobasTaqScreen MPX assays are carried out by single enzyme Z05 DNA polymerase. TMA probes are labelled with acridinium ester but in other case dual labelled with reporter and quencher dye. TMA is nearly isothermal but thermal cycling occurs in PCR. In TMA amplification of RNA templates occurs while in PCR amplification of DNA templates occurs [12-16].

Detection of Genotypes of HBV, HCV and HIV

ProcleixUltrio, Ultrio plus assays claim to detect HIV-1 group M (subtypes A, B, C, D, E, F, G& H), group N and group O genetic variants. In case of HCV, genotypes 1 to 6 and in case of HBV genotypes A to G [12,13]. ProcleixUltrio Elite assays claims to detect HIV-2, HBV genotype H, in addition to the claims of other Procleix assays [14]. CobasTaqScreen MPX assay claims to detect HIV-1 group M (subtypes A, AE, AG, B, C, D, E, F, G, H& J), group N, group O and HIV-2 (subtypes A, A/B, B). In case of HCV, genotypes 1a, 1b, 2, 2a, 2a/c, 2b, 3a, 3a/b, 4, 4a, 4b/c, 4h, 5, 5a, 6 and 6a and for HBV genotypes A to H are claimed to be detected [15]. In addition to the genetic variants detected by CobasTaqScreen MPX assay, CobasTaqScreen MPX v2.0 assay detects HIV-1 group M (subtypes B/D and G/BG), HCV genotypes 4c, 4acd, 4d, 4p, 4q, 6a/b and 6c [16].

Result Reporting

The cobasTaqScreen MPX assay and all Procleix assays report status of specimen as reactive if the target has been detected either for HBV or HCV or HIV or all [12-15]. Once the specimen is reported as reactive, the presence of individual virus is determined separately with individual virus detection assays. But, cobasTaqScreen MPX version 2.0 assay reports detection of individual viruses and there is no need for individual resolution testing [16].

Internal Control (IC)

Internal control is added to each specimen, control (negative & positive) and assay calibrator to monitor specimen preparation, amplification and detection procedures. IC is a target sequence (not related to HBV, HCV or HIV) and a calculated amount of it is added into the specimen. The detection of IC within the required range determines the validity and performance of the test. In Procleix assays IC is discriminated from HBV, HCV and HIV signal by the differential kinetics of light emission from probes with different labels. IC-specific amplicon is detected using a probe with rapid emission of light, while amplicon specific to HBV, HCV and HIV is detected using probes with relatively slower kinetics of light emission [12-14]. In case of cobas MPX assays different fluorescent dyes are used for IC, HBV, HCV and HIV probes, which are detected at different wavelengths [15,16].

Assay Calibrators

Procleix assays use negative calibrator, HBV positive calibrator, HCV positive calibrator and HIV positive calibrator. The relative light units (RLUs) detected from these calibrators are used to decide the validity of assay run and also to determine the status of specimen by determining the cut-off value. These calculations are performed by lunimometer [12-14].

CobasTaqScreen MPX assay uses negative control, positive controls for HBV, HCV and HIV, while in CobasTaqScreen MPX version 2.0 assay positive control for HBV and HCV has been combined in a vial and named as MPC. The cut-off for controls is determined on the basis of CT (threshold cycle) value. All controls should be valid for an assay run to be valid [15,16].

USER'S POINT OF VIEW

The comparative study for evaluating the performance of the cobas s201 and ProcleixUltrio Tigris systems indicated that ID-NAT was significantly more sensitive than testing in minipools of six donations in detecting HIV and HCV RNAs, but the difference in sensitivity for HBV DNA was limited. Overall both systems appear similar with regard to different quality features such as genotype sensitivity or analytical sensitivity. The cobasTaqScreen might be slightly more sensitive for HBV, while ProcleixUltrio detects lower levels of HCV or HIV-1 [19-21]. Chatterjee *et al*., has compared the sensitivity of individual donor and minipool NAT testing on Procleix assay system. The group observed that samples with high viral load were detected in minipools, but 67% of samples of low viral load were missed in minipool NAT. The study concluded that ID-NAT is ideal methodology because, in MP-NAT the dilution of NAT yield samples occurs [22]. The use of UltrioPlus NAT assay with increased 95% limit of detection of 3 IU/ml has doubled the yield of both window period and occult HBV infection detection [23]. In Hong Kong each system (ProcleixUltrio Tigris and cobasTaqScreen MPX s201) reported two different HBV NAT yield for a combined rate of 0.04%. The 95% detection limits for HBV, HCV and HIV-1 were 12.2, 2.0 and 42.2 IU/ml respectively, for Ultrio and 50.5, 8.4, and 6.0 IU/ml for the cobas MPX. The invalid test and failed run rates were 0.05% and 2.92%, respectively, for the Tigris and 2.39% and 5.53% for the cobas s201. It concluded that clinical sensitivity for HBV in Hong Kong blood donors was equivalent, as was the analytical sensitivity for HIV-1 and HBV. However, the ProcleixUltrio assay had a higher analytical sensitivity for HCV [24]. The comparisons of MPX-MP6 versus Ultrio Plus-ID demonstrated no difference in detection of NAT-yield samples for any of the three viruses [19,24-27]. During the screening of 486676 seronegative blood donations the analytical sensitivity of both systems the ProcleixUltrio-Tigris and the cobasTaqScreen MPX-s201 met the 95% limits of detection claimed by their respective package inserts. The test specificity for ProcleixUltrio and cobasTaqScreen MPX were 99.93 and 99.90 respectively. The NAT yield for HBV, HCV and HIV-1 were 1:2800, 1:490000 and 1:97000 respectively. Several occult HBV donors were also identified and majority of them were detected by both systems. The HCV and HIV-1 window period cases were detected by both tests. However, cobasTaqScreen test appeared to be more sensitive than ProcleixUltrio for HBV. The cross contamination was not observed in any system [25]. The American Red Cross implemented automated multiplex NAT for HBV, HCV and HIV in June 2009. Stramer et al., compared the relative sensitivity of these two United States Food and Drug Administration-licensed NAT systems for detecting HBV-infected donors in minipool sizes (MP) in the United States. The cobasTaqScreen MPX MP6 (minipool of 6 individual donors) was found more sensitive than Ultrio MP16 (minipool of 16 individual donors), but the impact of this difference was said to be less significant due to low numbers of HBV WP infections in United States [18].

CONCLUSION

A diagnostic assay should be sensitive, specific, user-friendly, rapid, robust and affordable. Automated triplex NAT assays are highly sensitive, specific and robust. But these assays required highly skilled manpower which is scanty in India. Also, the cost of equipment and reagents is very high. In developing country like India, it will remain out of reach to a large population.

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