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METHODOLOGY CONSIDERATIONS FOR THE MOLECULAR DETECTION OF TORCH ORGANISMS USING PCR APPROACHES

Bhumika Prajapati¹ , Kranti Vora¹ , Aarthi Sundararajan¹, Shahin Saiyed¹, Ruchi Tiwari² ,
Kuldeep Dhama³ , Senthilkumar Natesan¹

¹Indian Institute of Public Health Gandhinagar, Lekawada, Gandhinagar, Gujarat, 382042, India

²Department of Veterinary Microbiology and Immunology, College of Veterinary Sciences, Deen Dayal Upadhyay Pashu Chikitsa Vigyan Vishwavidyalay Evum Go-Anusandhan Sansthan (DUVASU), Mathura, 281001, India

³Division of Pathology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, 2431224, India

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ABSTRACT

There is a need for a reliable and cost-effective molecular diagnostic assay for the diagnosis of TORCH [*Toxoplasma gondii*, **O**ther (Varicella-Zoster virus and Parvovirus B19), **R**ubella virus, Cytomegalovirus, and **H**erpes Simplex virus] infections. This would enable early and precise detection of pathogens even in a very low copy number. However, the selection of genomic target, specimen matrix, and different PCR methods can significantly affect the sensitivity and specificity of TORCH molecular detection. This review aimed to provide a comparative analysis of clinical sample types, target nucleotide sequences, and PCR detection approaches for molecular detection of TORCH organisms. This review will aid in the development of a sensitive molecular assay for quick and precise detection of TORCH organisms.

* Corresponding author

E-mail: snatesan@iiphg.org (Senthilkumar Natesan)

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1 Introduction

TORCH is a group of infectious organisms including *Toxoplasma gondii* (*T. gondii*), Other [Varicella-Zoster virus (VZV), Hepatitis B, and Parvovirus B19 (B19V)], Rubella virus (RubV), Cytomegalovirus (CMV), and Herpes Simplex virus (HSV) (Yadav et al., 2014; Prasoon et al., 2015; Neu et al., 2015; Cofre et al., 2016). This is commonly found in a fetus or newborn showing clinical features well-matched with a vertically acquired infection and requires coherent diagnostic and treatment approaches. Infections such as these during pregnancy are a major cause of congenital abnormalities and fetal damage or loss. However, the period of gestation during which infection occurs determines the degree of severity experienced by the fetus (Yadav et al., 2014; Neu et al., 2015). TORCH infections have an immense public health impact. In US alone, around 30,000 children (~1 in 150) are born with congenital CMV, a member of TORCH infections, each year and around 5500 of them experience some form of such as deafness, blindness, and intellectual disabilities (Dollard et al., 2007; Cannon et al., 2012). Several methods such as serology, enzyme-linked immunosorbent assays (ELISAs), direct agglutination tests, complement fixation tests, virus isolation, histopathology, and nucleic acid based molecular assays, are currently used to screen for TORCH infection (Paiva-Cavalcanti et al., 2010; Sen et al., 2012; Wang et al., 2015; Suviriyapaisa et al., 2017; Dominguez et al., 2018; Ahmadpour et al., 2019). The traditional "TORCH test" is no longer suitable and has been replaced by specific tests for specific pathogens in well-defined situations. In the recent era of genomics, molecular approaches have become an essential standard to detect specific organisms, monitor infections, and quantify viral loads.

Despite the potential for sensitive pathogen detection offered by molecular methods, there are several pitfalls limiting their optimal performance. PCR based methods depend on the selection of 'primers', which can affect the performance the assay depending on their nucleotide sequences, secondary structures, and the formation of undesired inter- or intra-molecular interactions. Additionally, PCR based methods also depend on the specimen type, nucleic acid extraction method, concentration of nucleic acids, presence of PCR inhibitors, PCR conditions, and specific type of PCR, i.e., conventional PCR (cPCR), nested PCR (nPCR), or real-time quantitative PCR (qPCR) (Rodrigues et al., 2013). For an efficient and accurate detection of any organism using PCR, specimen and primer selection is a crucial process (Petrik, 2001; Lorenz, 2012).

The development of in-house molecular assays for detecting TORCH infections requires deep theoretical knowledge of the causative pathogens and cautions to account for genomic variations that may affect the assay sensitivity and specificity. There is a substantial amount of work published on the detection of specific TORCH organisms individually by targeting different genomic regions from a variety of samples; however, there is currently no comparative

analysis of these approaches. Therefore, we attempted to comprehensively review the published work regarding molecular detection of TORCH organisms with respect to the clinical specimen, target gene selection, primer selection, and assay methodology. The aim of this paper was to review the development and validation of PCR assays for detecting TORCH organisms, including factors that can affect the overall assay sensitivity and specificity.

2 *Toxoplasma gondii*

2.1 Specimen selection

Toxoplasmosis must be diagnosed early during pregnancy to offer early treatment and reduce the possibility of congenital infection in the fetus (Emna et al., 2006; Ahmadpour et al., 2019). PCR detection of *T. gondii* is a rapid and sensitive method to detect pathogen DNA in a wide range of clinical or non-clinical specimens. Whole blood (WB), along with its components; i.e., serum, plasma, peripheral blood mononuclear cells (PBMCs), buffy coat, and other bodily fluids, including amniotic fluids, can be tested using PCR. However, there is no consensus on the optimal sample for use in *T. gondii* detection (Contini et al., 2006). The French National Reference Centre for toxoplasmosis has advised the medical community on proposing guidelines regarding sample selection for molecular detection methods (Sterkers et al., 2010; Varlet-Marie et al., 2014). Molecular detection of circulating parasite DNA is beneficial for serology-based diagnosis. Research in animal models has preferred using the buffy coat over WB and has focused on the importance of blood volume for increasing PCR sensitivity. A 15-fold increase in sensitivity was observed with buffy coat isolated from a higher volume (7 ml) of blood compared to WB, plasma, and buffy coat from a smaller volume (200 µl) of blood (Brenier-Pinchart et al., 2015). Most studies have evaluated the performance of the PCR assay using blood sample volumes of 5 or 10 mL (Khalifa et al., 1994; Dupon et al., 1995; Lamoril et al., 1996; Bourdin et al., 2014; Robert-Gangneux et al., 2015), although some used only 1 mL (Correia et al., 2010; Cardona et al., 2011). Samples with lower blood volumes may explain the poor sensitivity of PCR assays given the decreased number of nucleated cells in the sample (Akane et al., 1994; Ajzenberg, 2010). Additionally, WB contains more potential PCR inhibitors, with heme being known to be a severe PCR inhibitor (Akane et al., 1994). In contrast, the use of peripheral blood has been reported to provide good sensitivity detecting *T. gondii* DNA in pregnant women (Bin Dajem & Almushait, 2012). Positive maternal diagnosis should be accompanied by fetal diagnosis. *Toxoplasma* DNA amplification from amniotic fluid has been considered the most sensitive and safest method for prenatal diagnosis and has replaced direct sampling of fetal blood. It is also highly suggestive of acute infections acquired during gestation (Montoya et al., 2002; Nimri et al., 2004;). However, amniocentesis for PCR is not recommended for pregnant women with human immunodeficiency virus (HIV) infection due to procedural risks of fetal HIV transmission (Romand et al., 2001).

2.2 Target sequence selection

The presence of *T. gondii* in biological samples can be detected through PCR by targeting its genetic material. The selection of *T. gondii* DNA target for PCR is still debated because different reports have suggested variable sensitivities depending on the target. To achieve higher sensitivity, the target sequence should be present in multiple copies and should be species-specific. Numerous multicopy genes, such as B1, 529 bp repeat element (REP529), internal transcribed spacer (ITS-1), and 18S rDNA, are widely targeted for detection of *T. gondii* in biological samples (Jauregui et al., 2001; Cristina et al., 1992). The non-coding repetitive element of *T. gondii* (TGR1_E) has also been evaluated as a diagnostic PCR target (Robert et al., 1996; Cermakova et al., 2005) but was abandoned due to sequence heterogeneity. The major surface antigen, the single copy gene P30 (P30, X14080.1), is also targeted for toxoplasmosis detection (Howe & Sibley, 1995; Rinder et al., 1995; Wang & Yin, 2014). However, the sensitivity to detect the parasite varies with each target gene. The B1 and REP529 regions are the two most frequently used target sequences and multiple primers have been designed that can anneal to various locations of these genes (Burg et al., 1989; Homan et al., 2000). Sequence analysis of REP529 indicated a highly conserved nucleotide sequence across various isolates of *T. gondii*, and targeting REP529 resulted in a 10-fold increase in sensitivity compared to B1 (Veronesi et al., 2017). In one study, a REP529 targeted PCR assay showed significantly higher ($p < 0.05$) sensitivity than a B1-PCR assay across 16 positive samples (Reischl et al., 2003). Targeting REP529 has also been reported to be more sensitive than B1 and enabled increased performance of toxoplasma diagnosis in all tested biological samples (placenta, amniotic fluid, WB, aqueous humour, cerebrospinal fluid (CSF), and bronchoalveolar fluid (Cassaing et al., 2006). Another study compared targeting REP529 and B1 in 76 blood samples and found 31/69 (45.6%) positive samples using REP529 targeted qPCR, none of which were detected with the B1 targeting assay. This indicates the superiority of REP529 targeting for the diagnosis of toxoplasmosis (Belaz et al., 2015). REP529 and B1 targeted PCR assays were found to have detection limits of 8 (640 fg) and 64 (5.12 pg) tachyzoites, respectively, in blood samples from leukemic children (Fallahi et al., 2014).

In contrast, the B1 targeting assay has been reported to be more efficient than REP529 because portions of the REP529 sequence were found to be absent from the parasite's genome in 4.8% of human *T. gondii*-positive samples (Wahab et al., 2010; Bin Dajem & Almushait, 2012). The serologically negative samples from 150 pregnant women were found to be positive by B1 targeting PCR assay, establishing B1 as a sensitive and specific target with a detection limit of 12 tachyzoites (Bin Dajem & Almushait, 2012). Mousavi et al. (2016) reported parasite prevalence of 67.8% and 57.1% by targeting B1 and REP529, respectively, in diabetic

patients. A comparative study focused on the detection of *T. gondii* DNA using three primer sets targeting B1, 18S, and P30 found that primers targeting P30 and ribosomal DNA (18S) were able to detect 1 pg of *T. gondii* DNA whereas B1 targeting primers detected as little as 50 fg (single tachyzoites). Additionally, sensitivity was not compromised when using B1 primers in the presence of substantial amounts of human lymphocyte DNA (Jones et al., 2000). Recent research from south India investigating the detection of toxoplasmosis in a variety of animal tissue reported B1 PCR to be 100% sensitive and specific (Rajendran et al., 2018). Other studies have also found B1 to be a more sensitive and specific target than REP529 for toxoplasmosis diagnosis using both nPCR and qPCR assays (Pereira-Chioccola et al., 2009; Mesquita et al., 2010; Al-Hadraawy & Hadi, 2017). Due to unavailability of specific primers, one recent work analyzed a set of 685 sequences from different *T. gondii* strains and reported the GRA7 gene to be a suitable target for primer design and the sensitivity of the GRA7 targeting assay was found to be similar to REP529 targeting and 40-fold higher than the B1 targeting assay (Costa et al., 2016). The repetitive mobile genetic elements (MGEs), with 100–500 copies per cell, are also targeted for molecular diagnostics, but are not completely species-specific (Ossorio et al., 1991). Among all targets, B1 and REP529 appear to be the most sensitive targets for detecting *T. gondii* DNA and have been successful in ocular fluids and retinal sections (Wells et al., 2015). Therefore, these genomic sequences should be targeted in PCR based assays to achieve efficient detection of the parasite.

2.3 Selection of PCR approach

Along with the specimen type and target genomic sequence, the type of molecular method used, i.e., cPCR, nPCR, qPCR, multiplex PCR, and loop-mediated isothermal amplification (LAMP), can also affect the sensitivity of parasite detection (Su et al., 2010). Initially, cPCR based in-house and commercial kits were the only choice for molecular detection of TORCH infection in most laboratories (Lavraud et al., 1995). Later, nPCR was introduced to increase the sensitivity of molecular detection. However, over the past few years, real time qPCR, with its ability to quantify parasite levels in clinical samples and monitor the infection, has been recognized as the most sensitive and specific molecular method for TORCH detection (Edvinsson et al., 2006; Djurkovic-Djakovic et al., 2012). The technical performance of three PCR assays, cPCR, nPCR, and qPCR, has been compared for the detection of *T. gondii* in non-immune pregnant women using B1 as the target gene. This showed that cPCR, nPCR, and qPCR detected 8, 11, and 12 cases of toxoplasmosis with a sensitivity of 67.0%, 92.0% and 100%, respectively, proving that qPCR is the most sensitive PCR assay (Aal et al., 2014). A comparison of four molecular assays observed a sensitivity of 84.7%, 98.3%, 96.6%, and 98.3 and efficiency of 91.0%, 95.0%, 98.0%, and 99.0% for cPCR, nPCR, multiplex nPCR, and qPCR, respectively in the diagnosis of toxoplasmosis in amniotic

fluid samples from 100 pregnant women. However, the same specificity, approximately 100%, was reported for all PCR methods (Teixeira et al., 2013).

In 64 amniotic fluid samples subjected to toxoplasma detection, the detection limit of cPCR was found to be 1000 parasites while real time qPCR with SYBR Green was able to detect 10-100 parasites. Real-time qPCR using hybridization probes based on fluorescence energy transfer (FRET) was able to detect single parasite and is thus considered the fastest and most sensitive method (Nagy et al., 2006). cPCR and qPCR assays were recently developed and evaluated to estimate a lower limit of detection and specificity when targeting B1 of *T. gondii*. By both methods, the lower detection limit for *T. gondii* DNA was found to be 10 pg, equivalent to 0.01 tachyzoites per μ l (Suviriyapaisa et al., 2017). LAMP is a nucleic acid amplification method that uses a polymerase enzyme with displacement activity and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA (Zhang et al., 2009). Comparing cPCR and LAMP identified detection limits of 10 and 1 pg, respectively, where 1 pg represents single *T. gondii* tachyzoites (Lau et al., 2010). LAMP assays have been reported to be 10 times more sensitive than nPCR, with a detection limit of 0.1 tachyzoites for detecting *Toxoplasma* DNA (Nagamine et al., 2002). The increased sensitivity and specificity of LAMP can be attributed to the increased number of primer pairs, which target six divergent regions of the target DNA (Shen et al., 1993).

3 Human Cytomegalovirus (HCMV)

3.1 Specimen selection

Sample type is an important preanalytical variable for detecting HCMV DNA. The virus can be detected in different fractions (cellular and cell-free) of blood and other bodily fluids, including urine, CSF, semen, vaginal secretions, umbilical cord blood (UCB), amniotic fluid, and throat washes (Razonable et al., 2001). However, the optimal specimen selection for CMV detection is still under the discussion. CMV is found to be excreted in multiple biological samples, but urine, blood, and amniotic fluids are the most commonly tested specimens. CMV establishes a lifelong latent infection following primary infection but may reactivate at any time owing to its shedding in urine, saliva, and other bodily fluids (Deback et al., 2007; Zhou et al., 2007). The peripheral blood lymphocytes have been reported for their increased sensitivity (Yerly et al., 2007), but their use is limited in leucopenia.

WB with anticoagulant has been suggested to be the ideal specimen given its higher DNA yield (Ross et al., 2011); however, serum or plasma is preferred for their accepted analytical performance and easier sample processing (Li et al., 2003; Arora et al., 2010). Due to persistence of virus for a month after primary infection during pregnancy, it can be easily detected in peripheral blood samples

(Madrid et al., 2018; Martin Ramirez et al., 2019). Numerous studies have identified HCMV DNA in pregnant women and found a relationship to the outcome of congenital infection (Munro et al., 2005; Revello et al., 2011). Fabrii et al. (2011) and Kaneko et al. (2006) assessed cord blood sera, Revello et al. (2011) evaluated amniotic fluids, and Munro et al. (2005) used maternal urine samples for HCMV detection. During the active phase of infection, virus is released from white blood cells (leucocytes), reticuloendothelial, and endothelial cells into the blood plasma, thus plasma viral load can indicate active HCMV infections. However, its absence does not exclude an active infection because the assay has a low detection limit, or the level of viral infection could be low. In contrast, one report has suggested, in cases of high viral load, that DNA can be detected more frequently in WB than plasma or serum due to the strict cell-associated nature of the virus (Garrigue et al., 2008).

Each type of sample has its own advantages and limitations, hence specimen selection for laboratory setup remains unsolved. WB is easier to process due to absence of cell separation steps, but viral testing from WB is susceptible to amplification inhibition from degraded hemoglobin products (Loens et al., 2007). In contrast, HCMV DNA quantification is easy to perform in plasma, but is limited by a lack of sensitivity compared to WB (Boom et al., 2002; Von Müller et al., 2007). PCR amplification of the large and small amplicon of HCMV showed that HCMV DNA highly fragmented in plasma and serum of renal transplant recipients which does not necessarily indicate the amount of infectious virus present (Chen et al., 2014). An 8-fold increase in viral titer was observed with 500 and 4000 copies/ml in plasma and WB, respectively, from a cohort of 82 renal transplant recipients (Chou et al., 1992; Abedi et al., 2017). A study from UCB of 825 pregnant women reported that the type of blood components can affect the positive rate of HCMV diagnosis given that a higher PCR positive rate for HCMV DNA was found in PBL compared to plasma (Khare et al., 2004). Simultaneous quantification of HCMV load in WB, plasma, PBL, and PBMCs from 319 samples revealed a higher viral load in WB compared to other blood components. Due to the invasive nature of blood collection, some patients might avoid testing. To address this, a group of researchers investigated the use of serial PCR assays on maternal urine samples for HCMV detection. Urine PCR results were compared with cord blood serology results and showed that only 1 out of 609 (0.16%) women had viral DNA in the urine and subsequently developed seroconversion (Khare et al., 2004). Such clinical research indicates that WB is the favorable specimen compared to serum or plasma due to positive detection, higher viral load, and increased sensitivity for HCMV monitoring.

3.2 Target sequence selection

PCR is a rapid, sensitive, and versatile method for HCMV detection that is based on the amplification of nucleic acids and generally targets major immediate-early (MIE) and late antigen genes in well conserved regions

(Lengerova et al., 2007; Leruez-Ville et al., 2008). Target gene selection can be based on its presence in clinically relevant strains, number of copies present in various species, conserved nature, and growth stages. HCMV is known to be highly divergent in certain coding regions and undergoes frequent recombination (Chou et al., 1992). Due to sequence variability, very few conserved regions, such as MIE (M21295.1), UL54 (DNA polymerase catalytic subunit, AB329634.1), UL55 which encodes glycoprotein B (gB) (U66425.1), UL83 (KJ743149.1) which encodes 65 kDa phosphoprotein and TRL1/IRL11 (open reading frame), can be exploited as target genes. The gB gene (UL55) is known to contain less variable regions of the HCMV genome, except for two variable subregions (Shepp et al., 1996; Murphy et al., 2003; Li et al., 2003), and has been frequently used as a target for certain PCR assays (Hong et al., 2004; Nye et al., 2005). Despite its conservation, one study reported the failure of gB region amplification for HCMV quantification in 11% (6/54) of isolates compared to DNA polymerase region targeting due to sequence diversity in the C630T variant (Habbal et al., 2009). The average variation in viral load was found to be associated with the amplification of target genes used for HCMV detection. Primer sets targeting the gB region have been reported to show higher sensitivity and specificity for detecting viral DNA from standard strains and clinical isolates than those targeting the UL54, UL83, and TRL11 regions (Enan et al., 2011). In contrast, another study found that primers targeting immediate early and late CMV antigen genes had 93% sensitivity and 100% specificity in urine samples from newborns with congenital CMV infection (Demmler, 1991). PCR targeting of a 105 bp region of the MIE gene identified 32 (32.7%) positive blood samples from 98 renal transplant recipients with viral loads ranging from 200 to 42932 copies/ml, suggesting that the MIE region is a sensitive molecular target for HCMV detection (Hayden et al., 2012). PCR amplification of DNA polymerase for HCMV quantification yielded lower viral load ($4.49 \log_{10}$) compared to other target genes: immediate-early ($5.16 \log_{10}$; $p < 0.0001$), MIE ($4.79 \log_{10}$; $p = 0.0269$), and gB ($4.82 \log_{10}$; $p = 0.0359$) (Zhang et al., 2010). A comparative study focusing on three functionally important and highly conserved CMV genes, DNA polymerase, gB, and the IE1 or MIE region, found that primers targeting the DNA polymerase gene were more sensitive and had less sequence variation for viral DNA detection in 46 isolates than those targeting the MIE region (Tabata et al., 2016). However, a recent study reported the development of an in-house multiplex PCR assay targeting the MIE gene with a lower limit of detection of 150 copies/ml (Duraismy et al., 2018). These results suggest that targeting the MIE region is a sensitive tool for the quantitative assessment of HCMV in clinical isolates (Tabata et al., 2016; Paul et al., 2018).

3.3 Selection of PCR approach

PCR is a rapid, sensitive, and versatile technique to detect HCMV infection. qRT-PCR is typically used considering its superiority in quantifying the viral titer and reducing the probability of contamination through the use of closed system. nPCR utilizes two

amplification rounds to significantly increase specificity and sensitivity. However, which is more suitable for HCMV infection monitoring is still under debate given that the results of many appropriate reports are contraindicated (Ikewaki et al., 2003; Botero et al., 2008). Zhang et al. (2010) compared two PCR techniques, qPCR and nPCR, for detecting HCMV DNA in 106 blood samples from 66 patients with potential HCMV infections. nPCR demonstrated a higher parasite detection rate than qPCR (34.9% vs 12.3%, $p < 0.001$) and nPCR was found to be the most sensitive method for HCMV detection. However, false positive results caused by cross-contamination remain a major barrier with nPCR. Therefore, one must consider strict adaptation of precautionary measures to limit this (Tabata et al., 2016).

To eliminate the problem of contamination in nPCR, a group of researchers have developed the boosted nPCR. This enables amplification in a single step and thus reduces contamination while achieving a detection limit of 1 fg of HCMV DNA, which corresponds to approximately 70 viral copies (Borg et al., 1995). Real-time PCR assays for quantifying HCMV have traditionally used TaqMan or hybridization probes (Nitsche et al., 1999). TaqMan probes are linear and labelled with a reporter and a quencher dye while molecular beacons are single-stranded oligonucleotide probes with a stem-loop structure. Real-time qPCR assays using TaqMan probes or a molecular beacon were developed for the detection and quantification of EBV and HCMV in blood samples from AIDS patients and both assays were reported to be reliable and reproducible with low interassay and intraassay variation. Moreover, these assays are suitable for routine diagnostic testing, but designing molecular beacons is more time consuming than TaqMan probes (Jebbink et al., 2003). A comparative study between real time qPCR and nPCR for the detection of HSV-1, EBV, and HCMV DNA indicated an overall agreement between the two methods, but one negative sample was found to be positive through real time PCR (Drago et al., 2004).

In contrast, one study reported improved detection of HCMV DNA using nPCR compared to real-time qPCR ($p < 0.0001$) in gingival crevicular fluid from a group of 44 periodontitis patients. The previous study mentioned that LAMP is highly sensitive method with a lower detection limit of 10 copies of HCMV DNA per microliter and has a good correlation with real time qPCR (Reddy et al., 2010). One study, using serially-diluted CMV gB-containing plasmids to determine sensitivity, revealed that greater than 10 copies per reaction were detectable using the HCMV LAMP method on blood and amniotic fluid samples from pregnant women (Wang et al., 2015).

4 Rubella Virus (RubV)

4.1 Specimen selection

Several sample types, such as WB, plasma, amniotic fluid, and urine, are used for rubella virus RNA detection. However, pharyngeal

samples, including nasal aspirates and throat swabs (TS), are the preferred specimens for detecting the rubella virus in both acute cases and congenital rubella syndrome (CRS) (Zhou et al., 2007; Yasui et al., 2014). Blood has been suggested not to be a good specimen for RubV detection because the virus remains undetectable in blood for two days after rash onset (Zhu et al., 2007). In contrast, the viral titer in throat swabs peaks on the day of rash onset and declines more slowly than in the blood, enabling virus detection up to five to seven days after rash onset (Davis et al., 1971; Zhu et al., 2007). However, most published reports have used peripheral blood or serum samples with good sensitivity for rubella RNA detection in pregnant women (Curti et al., 2014; Zanga et al., 2017). Along with the sample type, collection timing is also a crucial factor. The sample should be collected immediately after symptom onset; between one to three days, but not after seven days, after the onset of symptoms to detect cases of acute rubella. Rubella virus continues to shed up to a year after birth in CRS cases. However, around 50% of patients will no longer shed virus and thus samples should be collected before 3 months of age (Sugishita et al., 2016).

4.2 Target selection

The RubV genome is comprised of less than 10,000 nucleotides which encode five proteins, including three structural proteins, E1, E2, and the capsid protein. The E1 (NC_001545.2) sequence between 8731 and 9469 nucleotide position in the RubV genome is recommended by the WHO as a target site for detection by PCR (WHO, 2005). Bosma et al. (1995) first reported primers targeting the E1 open reading frame to be a sensitive and specific strategy for RubV detection by reverse transcription nPCR (RT-PCR). This approach was able to detect two RNA copies from five wild-type rubella strains and four vaccine strains with no nonspecific amplification of 16 other RNA viruses. The complete nucleotide sequence of E1 in RubV is highly conserved across various wild-type rubella strains and is also likely present in most clinical samples infected by RubV (Dominguez et al., 1990). A number of studies described the phylogenetic analysis of 103 different E1 gene sequences from RubV isolated from 17 countries between 1961 and 2000 and concluded that the E1 gene region was the most highly conserved target among all strains (Katow et al., 1997; Zhou et al., 2007). Many published reports have targeted the E1 open reading frame for qualitative or quantitative determination of RubV from a variety of clinical specimens, including from pregnant women, and described it to be a specific and sensitive approach for molecular assays (Vyse & Jin, 2002; Lazar et al., 2016).

4.3 Selection of PCR approach

Several studies have reported the development of reverse transcription nPCR (RT-nPCR) and qRT-PCR assays for detecting RubV RNA using the oligonucleotide pair targeting a conserved region of the E1 gene (Katow et al., 1997; Zhou et al., 2007). One study reported the positive samples from oral fluid (13/19; 68.4%), throat swabs (26/41;

63.4%), and fetal tissue (2/2; 100%) to find a total positive sample rate of 66.0% using RT-nPCR for detecting rubella virus (Reddy et al., 2010). Others have reported that the optimized nRT-PCR assay was specific and sensitive enough to detect just 10 fg of rubella RNA (Shyamala et al., 2007). Okamoto et al. (2010) developed a high throughput TaqMan based one step real time assay, and its sensitivity was compared to conventional RT-nPCR for the diagnosis of rubella infection. They found the qPCR assay to have a detection limit of 10 copies of rubella RNA and 1 pfu of virus and considered it to be useful for rapid screening and diagnosis of rubella in conjunction with nPCR (Okamoto et al., 2010).

5 Herpesvirus Family (HSVs & VZV)

5.1 Specimen selection

Different specimens, such as cerebrospinal fluid (CSF), WB, serum, plasma, and cellular samples including biopsies, swabs, bone marrow etc., are used for the molecular detection of HSVs DNA. Paraffin-embedded tissues can be also tested for the presence of virus after deparaffinization. The optimal sample for viral detection depends on the virus and its associated clinical disease. Gestational tissues from 36 women with pregnancy loss were tested for HSV1-2 DNA by nPCR (Kapranos & Kotronias, 2009). Most reports have mentioned the use of cervical smears (Dinc et al., 2010; Caldeira et al., 2013) and blood (Zhou et al., 2015) samples for HSV1-2 DNA detection in pregnant women using a PCR approach. One group tested WB and serum or plasma specimens from 21 patients with clinical symptoms of varicella and 21 with herpes zoster and reported the lower limit of detection sensitivity to be 20 copies/ml from plasma or serum and 80 copies/ml from WB. This shows that the quantification of VZV or HSV DNA in WB has potential for the diagnosis and clinical management of infected patients. In contrast, cell-free plasma and serum provide convenient matrices for this purpose (de Jong et al., 2000). The presence of virus during VZV infections is thought to be highly cell-associated and this has been observed during *in vitro* growth cycles of the virus. However, the quantification of HSVs viral DNA in blood has been restricted to the analysis of isolated PBMCs (de Jong et al., 2000).

5.2 Target selection

Detection of HSV1-2 DNA by PCR is a classical molecular approach routinely performed by numerous clinical laboratories. The similarities and variations between HSV1-2 genomes are utilized for genera specific molecular assays. The gB and gD genes are targeted to differentiate HSV1-2, while the DNA polymerase gene is targeted for detecting both serotypes simultaneously (Arvin et al., 1996; LeGoff et al., 2014). The major seven loci, distributed throughout unique long (U_L) and unique short (U_S) regions of the HSV1-2 genomes, include DNA polymerase (HSV1: X04771.1, HSV 2: M16321.1), gB or UL27 (AF259899.1, AF021340.1), and thymidine kinase (TK) or UL23 (HSV1: AB009254.2, HSV2: AB009257.1)

genes which are widely reported for the detection of viral DNA in various clinical specimens (LeGoff et al., 2014). The detection of HSVs DNA by targeting the TK gene in CSF samples revealed its highly variable nucleotide sequence. However, analyzing a 335-bp amplicon within the TK gene revealed the presence of 51 nucleotides that are consistently present to enable differentiation between HSV 1-2 (Aslanzadeh et al., 1993; Tang et al., 1999). Unlike TK, the DNA polymerase gene comprises highly conserved region and degenerate primers targeting this region are used to amplify DNA from several species of human and animal HSVs. HSVs DNA quantification by targeting the DNA polymerase gene has been reported to have detection limits of 0.5 fg, corresponding to the 3.1 viral particles for HSV-1, and 0.2 fg, corresponding to 1.3 viral particles for HSV-2, indicating good sensitivity (Madhavan et al., 1999).

5.3 Selection of PCR approach

Qualitative PCR assays, such as nPCR and PCR, along with southern blot hybridization are widely used for the diagnosis of HSV infection. However, quantitative assays based on real time PCR are more recently used to measure the HSV viral load (Kimura et al., 2002). A recent study suggested the nPCR method to be better than the virus culture assay (Dominguez et al., 2018). Kawada et al. compared qPCR with nPCR and reported that real-time qPCR had a specificity of 99% (137/139) and sensitivity of 100% (25/25) compared with nPCR in detecting 100 copies per ml from CSF samples (Kawada et al., 2004). While testing 110 dermal or genital lesions, the nPCR assay was reported to detect 22%, 37%, and 46% of samples positive for HSV-1, HSV-2, and VZV, respectively, whereas, qPCR detected 24%, 36%, and 46% of samples positive (Schmutzhard et al., 2004). The testing of 46 samples using nested and real time PCR targeting the gpD region showed the same number of positive samples by both assays; however, one negative sample in nPCR was detected as positive in qPCR. Thus, qPCR demonstrates comparatively higher sensitivity and specificity (Aslanzadeh et al., 1993). A comparative analysis between cPCR and real-time PCR in 147 CSF samples was performed for detection of major neurotropic viruses, i.e., HSV1-2, CMV, VZV, and EBV. Overall, real time PCR yielded 88 (59.9%) positives while cPCR had only 6 (4.1%), suggesting qPCR is much more sensitive than cPCR (Ramamurthy et al., 2011). A significant ($p < 0.05$) increase in positive samples was detected by real-time qPCR (44%) compared with (8%) nPCR (Franzen-Rohl et al., 2007). The lower limit of detection for an in-house developed nPCR assay was reported to be approximately 5 copies/ μ L for HSV and 10 copies/ μ L for VZV, indicating high analytical sensitivity (Rodrigues et al., 2013).

6 Parvovirus B19 (B19V)

B19V infection during pregnancy can infect the fetal erythroid precursor cells and fetal tissues, which may act as a danger signal for

the fetus. B19V is the smallest DNA virus that can be detected in the plasma, serum, peripheral blood, amniotic fluids, and cord blood, and was first identified in the plasma in 2005 by random molecular screening (Allander et al., 2005; Naciute et al., 2016). The two distinct viral proteins, named viral capsid proteins VP1 (83 kDa) and VP2 (58 kDa), and a non-structural (NS1, AY903437.2) protein are encoded by regions widely targeted for B19V detection. Chen et al detected B19V DNA by targeting the VP2 gene and were able to detect as few as five copies of viral DNA per reaction using PCR (Chen et al., 2009). In contrast, several studies have targeted the NS1 region in serum samples of pregnant women and reported it to be a highly specific molecular target for detecting B19V DNA (Shabani et al., 2015; Arabzadeh et al., 2017).

An overview on the molecular detection of TORCH organisms and their target nucleotide sequences for PCR detection is depicted in **Figure 1**.

Conclusion and Future Directions

We have evaluated the key factors and variables that can affect the sensitivity and specificity of molecular detection of TORCH organisms. Variation in target genomic sequences, specimen matrix, primer sequences, and modifications to PCR conditions can significantly affect the performance of molecular assays. Despite the broad use of conventional and nested PCR for TORCH detection, their application is limited to only qualitative measures. In contrast, real time PCR provides both qualitative and quantitative measures and increases the limit of detection while reducing the chances of contamination. After analyzing published reports, buffy coat has been found to be a reliable specimen matrix for PCR detection of TORCH organisms. The buffy coat can yield sufficient amounts of DNA, even after extended periods of storage. It offers the advantage of increased numbers of blood cells, and thus, higher copies of the organisms owing to the cell-associated nature of most TORCH organisms. The target genomic sequence should be present in the most conserved regions of all strains and should be in multiple copies to achieve efficient molecular detection. After a thorough review of the published reports, we can understand that genomic regions such as B1, major immediate early region, DNA polymerase, and E1 protein are the ideal targets for efficient detection of toxoplasma, cytomegalovirus, herpesvirus, and rubella, respectively, using PCR assays. The principal factors affecting the molecular detection of each organism of TORCH should be thoroughly understood before applying them for clinical detection. The development of a highly sensitive and specific multiplexed PCR assay to detect all the organisms of TORCH will be a quick and cost-effective approach that should be developed in future. It will be highly useful for screening large numbers of pregnant women in public health studies and suggesting preventive or curative measures.

Figure 1: Molecular detection of TORCH complex organisms and their target nucleotide sequences for PCR detection. The figure showing the complete genome structure of TORCH organisms. There are multiple PCR targets such as B1, REP529, P30, 18s rRNA for Toxoplasma; immediate early genes, thymidine kinase, DNA polymerase and various glycoproteins for Herpesvirus family while viral capsid proteins (VP1 and VP2) and non-structural proteins for Parvovirus B19 detection. Rubella virus can be detected by targeting the highly conserved envelope glycoprotein region.

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Author contributions

All the authors substantially contributed to the conception, compilation of data, checking and approving the final version of the manuscript, and agree to be accountable for its contents.

Conflict of interest

All authors declare that there exist no commercial or financial relationships that could, in any way, lead to a potential conflict of interest.

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