A review of performance of Zika assays in the context of TORCH diagnostics

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Running title: **TORCH and perinatal Zika diagnostic challenges**

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SUMMARY

Infections during pregnancy that may cause congenital abnormalities have been recognized for decades, but their diagnosis is notoriously challenging. This was recently again illustrated with the emergence of Zika virus (ZIKV), highlighting the inherent difficulties in estimating the extent of pre- and postnatal ZIKV complications, because of the difficulties in establishing definitive diagnoses. We reviewed the epidemiology, infection kinetics and diagnostic methods used in *Toxoplasma gondii*, Parvovirus B19, Rubella virus and Cytomegalovirus (TORCH) infections and compared that with current knowledge of ZIKV diagnostics to provide a basis for the inclusion of ZIKV in the TORCH complex evaluations.

Similarities between TORCH pathogens and ZIKV support inclusion of ZIKV as an emerging TORCH infection. Our review evaluates diagnostic performance for maternal screening, fetal screening and neonatal screening. We show that sensitivity, specificity, and positive and negative predictive value of TORCH complex pathogens are wide widely variable, stressing the importance of confirmatory testing and the need for novel techniques for earlier and more accurate diagnosis of maternal and congenital infections. In this context it is also important to acknowledge different needs and access to care for different geographic and resource settings.

INTRODUCTION

The present Zika virus (ZIKV) epidemic was first noted through an alert from the Brazilian health authorities notifying the World Health Organization (WHO) of an illness characterized by skin rash in north-eastern states and subsequently signalling an almost 20-fold increase in

the incidence of microcephaly in newborns coinciding with rapid spread of ZIKV after incursion into the continent [1,2,3,4]. Until then, ZIKV infection was generally assumed to be associated with mild and transient disease, estimated to be asymptomatic in approximately 80% of the cases [5]. Hence, it is likely that the infection is underdiagnosed or underreported in a disease-endemic setting [6].

The association of ZIKV infection with congenital neurological disease has since then been subject of numerous publications. First establishment of ZIKV causal association with neuropathological processes came from a study showing widespread ZIKV infection in the brain of a fetus from a pregnancy that had been terminated due to severe fetal malformation [7]. Further evidence for the association came from larger case series, retrospective analysis of notification data from regions with prior outbreaks [8], and replication of the syndrome in animal models [9,10,11,12,13]. Although there is general agreement of such association, many uncertainties remain with regard to actual risk of fetal infection during pregnancy [14]. A systematic review estimated the prevalence of microcephaly at 2.3% (95%CI: 1,0-5.3% [15], but estimates range widely and little is known about the risk of complications in relation to timing of maternal infection (first trimester versus later exposures), prior (flavi virus) exposure, the rate of transplacental transmission in relation to these factors, the rate of fetal infection, and the rate of congenital disease once infected (Figure 1). Two recent reports suggest a decreasing risk over the course of pregnancy [16,17]. A recent European study showed an overall congenital anomaly prevalence of any (non-genetic) cause of 1.5/100 total births, but for microcephaly interpretation was hampered by differences in diagnostic criteria [18].

At present, ZIKV diagnostic algorithms are based on the use of reverse transcriptase-polymerase chain reaction (RT-PCR) for virus detection and/or serological determination of pathogen-specific IgM and IgG antibodies supplemented with virus neutralisation assays if

available. All of the assays have benefits but also known limitations, challenging interpretation at different stages of pregnancy, particularly in relation to the wide diversity of flavivirus background exposures in the regions where ZIKV circulates. Virus genome detection by RT-PCR is considered confirmatory but has a very short detection window as ZIKV viremia is short, although virus may persist for longer periods in other body fluids, with reported persistence up to 120 days for semen [19]. Also pregnant women may experience prolonged viremia [20,21], with reported (transient) presence of ZIKV in fetal blood and amniotic fluid described in a well described small case series [21]. Antibody based testing is severely hampered by cross-reactivity with antibodies from prior flavivirus exposures.

Other infections during pregnancy are associated with congenital and subsequent neonatal disease, sometimes referred to as TORCH infections (*Toxoplasma gondii* (TOXO), Other [e.g. varicella zoster, parvovirus B19 (PB19)], Rubella (RV), Cytomegalovirus (CMV) and Herpes simplex (HSV) with or without syphilis [22]. Diagnosis of fetal infection and linking fetal infections to clinical outcomes requires knowledge of infection kinetics, including timing and differentiation of primary from nonprimary infection (i.e. re-activation, re-infection), maternal and fetal immune response in relation to pathology, and availability of biomarkers predictive of vertical transmission and presence and/or severity of fetal abnormalities [23,24,25]. For instance, TOXO and CMV cause persistent or latent infections, whereas RV, PB19 and ZIKV infection are thought to be primarily self-limiting. Immunocompetent pregnant women with previous infection with TOXO are considered not at risk for congenital abnormalities, whereas for CMV primary as well as subsequent infections are associated with congenital infection and abnormalities, albeit with a lower attributable risk. The recent ZIKV epidemic and its possible association with microcephaly has initiated the discussion to include ZIKV as novel TORCH pathogen [26].

Although the described maternal infections are an important cause of fetal and neonatal morbidity and mortality, on a global scale, the overall contribution to fetal and congenital diseases is limited [27,28,29] due to cumulative low and/or limited a priori risks of maternal infection, vertical transmission and subsequent congenital infection (Figure 1).

Generally, risk of congenital disease following maternal infection is linked to primary infection and timing in pregnancy infection, which is pathogen dependent [25,30,31,32,33,34,35,36,37]. Non-primary maternal infections may result in fetal transmission, but only in the case of CMV does this contribute significantly to congenital disease [38].

The low attributive risk of TORCH and ZIKV infections to congenital disease has consequences for diagnostic accuracy and the ability to provide information relevant for clinical decision-making. This is further complicated by the high proportion of asymptomatic maternal infections [6,35,39,40,41], challenging timely detection of fetal infection and early neonatal congenital disease, which may remain asymptomatic for years [40,42]. Early diagnosis of fetal disease risk in pregnancy, however, is important particularly when early therapeutic management is available eg. in TOXO [43]. Consequently, diagnostic algorithms should reliably and timely detect maternal infection, determine (risk of) vertical transmission and establish or exclude congenital infection. This review assesses diagnostic methods presently used for TORCH infections, their correlation with congenital and/or neonatal disease, their predictive value in prenatal screening; it will document gaps in methods used; and it will draw implications for diagnostic algorithms d evelopment in novel or (re)emerging infections such as Zika virus.

EPIDEMIOLOGY, INFECTION AND TRANSMISSION RISKS OF SELECTED TORCH PATHOGENS (FIGURE 1)

The risk of infection during pregnancy varies by pathogen and depends upon geographic region, prevalence in the population, and preventative (vaccination) practices.

The seroprevalence of *Toxoplasma gondii* among women of childbearing age shows a broad range, from <2% in a large Chinese cohort [44] up to 75% in Brazil [45], with a mean estimate of around 40% [46,47,48]. Similar broad ranges in seroprevalence between 30-72% are reported for PB19 and CMV [49,50,51,52,53,54,55,56] although for CMV seroprevalences up to 100% are also reported [57]. RV seroprevalence estimates depend on (differences in) vaccination practices [58,59,60]. ZIKV seroprevalence has a geographic distribution, varying from <2% in travellers returning from endemic areas or blooddonors in non-endemic settings [61,62,63] to up to 39% in healthy individuals living in endemic areas [64,65] increasing to >60% following outbreaks [66, 67]. These wide ranges in background seroprevalence affect the likelihood of primary infection during pregnancy, as well as the interpretation of diagnostic assays, and need to be taken into account when developing diagnostic algorithms.

To assess clinical impact of exposure to TORCH pathogens and ZIKA during pregnancy, it is important to consider maternal infection risk, fetal transmission risk, and congenital infection risk for each pathogen (Figure 1). Maternal infection risk (MIR) estimates defined as the annual infection rate for selected TORCH infections, range from 0.1-0.6% for TOXO [31] to 2-7% for PB19 and CMV [68,69,70,71,72,73], with epidemic rise up to 10% in PB19. Reliable data for RV MIR is lacking in an elimination setting, but annual incidence is estimated at 1.3/100.000 pregnancies in the general population [74]. A 6.4% IgG seroconversion was reported in women with non-immune RV titers prior to pregnancy [75].

Although efforts have been made to calculate the maternal infection risk for ZIKV, reliable data are still lacking, due to several factors including diagnostic limitations (e.g. cross-reactivity) [76,77], and difference and rapid changes in epidemiology.

Fetal transmission risk (FTR), defined as the proportion transplacental transmission following (primary) infection during pregnancy, is also pathogen dependent and is linked to timing of infection during pregnancy. FTR may increase (TOXO, PB19, CMV) or decrease (RV) during the pregnancy period, with a variable mean FTR estimated to range from 24-80% for these pathogens [25,33,42,78,79]. For ZIKV, FTR is thought to be highest during first trimester, but more data is needed [16,17]. Perinatal transmission has also been reported for ZIKV [80].

Despite high FTR, the congenital infection risk (CIR) defined as number of congenital infections per 1000 live births (or number of fetal deaths/hydrops fetalis per 1000 infeced fetuses in PB19) is low, ranging from <1/100000 pregancies (RV) up to 0,1-20/1000 pregnancies in TOXO, PB19 and CMV [33,34,81,82,83,84,85,86,87,88,89]. Overall congenital CMV infection is most prevalent in the developed world (5-20/1000 live births) [30,90], followed by toxoplasmosis (0.1-5/1000 live births) [31,46], and RV (annual incidence 0.4/100.000)[81]. Parvovirus B19 is associated with hydrops fetalis [82,83,84] and fetal death, with an estimated annual incidence of <4/1000 fetuses [85]. Recent studies estimated the contribution of symptomatic ZIKV during pregnancy to ZIKV associated congenital disease at 7% [17], and evidence of acute infection in pregnancy at less then 4% [77]. How this translates to the overall contribution of ZIKV to e.g. congenital microcephaly prevalence depends on the baseline risk and these are uncertain [18,91]. A retrospective analysis in French Polynesia estimated a risk of microcephaly associated with ZIKV infection at 9.5/1000 pregnancies, with an overall risk of microcephaly in 0.2/1000 neonates [8].

CIR does not necessarily follow the FTR, with highest CIR in the 1st trimester for TOXO, RV and CMV [25,35,34,42] and highest CIR in the second trimester for PB19 [36,37]. Also, for ZIKV highest risk is reported in 1st trimester [8,17]. Overall CIR is limited to primary infection, except for CMV, where re-infection or re-activation contributes mainly to congenital CMV disease burden [56,92]. Latent or chronic TOXO infection does not exclude transmission but does not result in CIR in non-immunocompromised pregnancies [93,94]. In general, the low attributive risk of the reported infections to overall prevalence of congenital disease [27,28,29] impacts on the performance of diagnostic assays.

This implies that the low a priori attributive risk of TORCH and ZIKV to congenital infections needs to be included in every step excluding or confirming maternal, fetal and/or congenital infection.

MATERNAL DIAGNOSTIC TESTING (FIGURES 2A – 2D)

Infection kinetics

Interpretation of diagnostic testing during pregnancy requires knowledge on infection kinetics defined by prevalence and duration of symptoms, duration of pathogen presence in different body fluids, loads, timing of development of specific antibodies, background antibody prevalence, and relationship between these parameters. Molecular detection in early symptomatic infection is generally considered proof of acute primary infection [17,40,59,95], although not true for each pathogen, i.e. CMV. Primary infections typically show IgM and IgG development, determined with serological assays with or without confirmatory testing [96,97]. Reported antibody kinetics differ between selected pathogens. TOXO IgM seroconversion occurs relatively late between 15-30 days [98,99], whilst early IgM rise is observed for PB19 towards the end of the first week of infection coinciding with peak viremia [56] as well as for RV whose IgM rise within 5 days after rash onset [100]. CMV IgM may

become detectable between 0-3 weeks [101] with peak IgM observed between 1-3 months [102]. IgM antibodies against ZIKV also show an early rise and can be first detected within the first week after clinical symptoms, but also IgG antibodies can be detected within the first two weeks [103].

PB19 IgM can persist up to 3 months postinfection [104], and ZIKV IgM can also persist beyond 3 months with a reported wide range [105]. In addition, long-term persistence of rubella IgM is reported following vaccination [106,107,108], due to natural occurrence of non-specific IgM [109,110] and despite attempts to improve assays [111,112,113,114]. Differentiation of acute infection from latent infection or re-activation / re-infection is important in TOXO and CMV, as IgM/IgG may coincide thereby making it difficult to diagnose primary infection if first consultation yields an IgM/IgG positive test result [115]. In this case, confirmatory testing is needed, e.g., by AI or IB with presently available assays. [47,116,117]. Development of CMV specific IgG with a negative sample collected earlier in pregnancy is considered proof of primary CMV infection, although in absence of routine screening this is usually not feasible [118].

Molecular assay performance and limitations

Although PCR assay specificity is high in acute primary infection, the window of PCR positivity may be short as shown for PB19 [95] [Figure 2]. Limited data from ZIKV showed a similar pattern. A recent external quality assessment (EQA) suggested similar more robust specificity and variable sensitivity between labs [119]. Furthermore, most acute (primary) infections in pregnancy are asymptomatic, and day of infection is unknown precluding use of this gold standard test. In primary PB19 in pregnancy, high viral load in acute infection is associated with early positive IgM [39,56,120,121]. Long-term, low load, DNA (desoxyribo nucleic acid) persistence is observed following PB19 infection [104,122]. In one study, the

use of endonuclease treatment before molecular testing differentiated naked DNA persistence from true viremia [123]. For ZIKV, rapid degradation of RNA (ribo nucleic acid) was reported in urine samples [124]. In acute maternal PB19 infection, positive predictive value (PPV) of PB19 PCR is high, but at time of fetal symptoms, the PPV of PB19 DNA detection in maternal blood is generally low, as clinical symtoms in the fetus are usually observed when maternal viremia has ceased. This timely relation is not established for other primary infections such as TOXO [125,126] or CMV [127]. Viremia in pregnant women is associated with vertical transmission risk and increased CIR but the relationship between maternal infection, FTR and CIR is different for different pathogens [128,129,130,131,132,133,134]. Currently, there is no obvious predictor for transmission risk. For instance, viral load does not differentiate transmitters from non-transmitters in CMV [129,130,135]. Absence of relationship with maternal disease severity or viral load was also recently described for congenital ZIKV [136]. Low viral load positives may occasionally not show IgM seroconversion (RV, ZIKV) [137,138,139], low assay sensitivity was suggested as one of the possible explanations [139]. Genotype differences may impact sensitivity of assays [140,141,142] which is important when considering using assays in different regions.

Serological assays and performance

Primary diagnostic assays

Generally, TORCH immunoassays report relatively high specificity for IgM and IgG or in IgM negative samples [34,108,113,143) but as the *a priori* likelihood of a maternal infection with TORCH pathogens generally is low, even a relatively low false positivity rate translates to a low PPV for all pathogens (including ZIKV) except PB19, stressing the need for confirmatory testing [144,145,146,147] [figure 2]. Comparative studies of assays, reporting relative performance data overestimate sensitivity and specificity [108,113, 143, 148]. In view

of the above, a positive IgM test result always requires confirmation with other assays [36,101,113,149] and follow up samples. More specific (recombinant) peptide specific IgM assays may provide solutions, but their performance also needs to be fully evaluated [121,150].

Confirmatory testing

The use of confirmatory testing with avidity index measurements (AI), immunoblots (IB) and virus neutralisation testing (VNT) is not consistent between pathogens and also show variable performances (Figure 2). Testing for AI is common practice for TOXO and CMV diagnostics, but not for PB19 [121] or RV [151]. The rationale for avidity testing is that avidity of antibodies increases with time, and high AI correlates with infection in the more distant past [152,153,154,155,156,157,158]. Confirmatory sensitivity of AI depends upon the initial screening platform used, as shown in one study. A negative initial IgM screening is unlikely to be confirmed [145]. IgM positivity combined with low AI increases sensitivity and PPV of the combined assays in diagnosing recent infection [159,160,161,162]. In contrast (persistent) low AI with positive IgG has a relatively high negative predictive value (NPV) [163,164]. High AI plus IgG usually confirms past infection, however, for TOXO AI maturation may never occur [165]. Rapid increase in AI in CMV was associated with false exclusion of recent infection [166] with higher FTR [167] and CIR [168]. Therefore, exclusion of acute infection based on (high) AI requires a predefined time window [101,114,169,170,171], and size of the window varies depending on pathogen, thresholds and platforms [172]. Different antigens, including recombinant antigens as target for antibody response may improve AI assay performance [170].

Antibody test results may also be confirmed by IB [101,173] or different complementary assays [174]. Epitope-specific IgG IB used in TOXO and PB19 could confirm IgM/IgG

measurements [175,176] particularly in equivocal outcomes [177] or in (false) negative results with high viral load [146] and helped timing of infection by correlation of IB with virusneutralisation in CMV [178] or with AI in TOXO [179].

An interesting application is the use of IB in TOXO for discrimination of maternal and neonatal antibody responses by comparing patterns of antibody binding to different proteins or peptides in blood from mother and neonate [180]. Limitations of IB include lack of standardisation with variable concordance between assays, particularly in acute infection [181,182] and different diagnostic accuracy of band patterns in the blots [175]. Predictive value of IB depends upon the target, and IgM blots often have poorer predictive value compared to IgG blots [173,183]. Virusneutralisation data are primarily available for CMV [96], but also commonly used in confirmation of ZIKV infection [184], with generally high assay performance.

Limitiations of serodiagnostic assays

A major limitation for all diagnostic methods described is interassay variability [98,185], use of different cut-offs, differences in classifications of positives [185,186], low agreement between AI index assays [187], variability between platforms [165,114,171,172,188,189,190,191] and lack of standardization. Another serious concern is the “grey zone” classification, i.e. the area between the negative outcome and the positive outcome of a test [98,114,153,163,164,166,186,187,192,193], which differs considerably between assays [194] and the lack of standardisation of cut-off values for the same assay [183]. Assigning the grey zone to the negative or positive group impacts on sensitivity or specificity (as indicated in figure 2, where for example category Tox+ denotes the assignment of grey zone to the seropositive group and Tox – assignment to the seronegative group) [148,195,196] stressing the need for common standards for assay development and validation

[197,198,199,200,201], use of a standardcurve [202] and/or (international) standardization as shown in RV [186,203]. Even when general standards for defining seropositivity are applied, different assays show different performance characteristics which is impacted by the assignment of equivocal results to the positive or negative outcome [108,204,205]. Since this mainly affects sensitivity it increases NPV, particularly when prevalence decreases [92,168,206,207,208].

FETAL INFECTION DIAGNOSTIC TESTING (FIGURES 3A – 3D)

In case of suspected fetal infection, molecular detection of virus DNA or RNA in amniotic fluid (AF) or cordblood (CB) is the primary diagnostic option in most cases except in TOXO. Limited serological data on AF include IgM determination [209,210]. IgG determination is not informative as it is usually of maternal origin. IgM and/or IgA determination in AF or fetal blood (FB) have low diagnostic value [211,212,213,214,215], whereas cell culture isolation (virological confirmation) is more specific for example in CMV [215]. Loads in FB or AF may be 100-1000-fold higher than in maternal blood [157,129,216], particularly in symptomatic fetuses [217,218,219] as shown in PB19, CMV and TOXO. Although (viral) load in primary infection may be high in the fetus [211] its presence is not necessarily associated with symptomatic infection [220,221,222,223]. Also, normal pregnancy outcome has been observed in maternal seroconversion without positive AF-PCR [208]. These discrepancies possibly reflect different windows of infection detection. Overall when available, PCR on AF or FB has good specificity and NPV in the fetus (Figure 3) [210,224,225,226,227,228,229] . For PB19, sensitivity has been shown to increase in presence of maternal viremia [216], and for TOXO with a shorter interval to AF or FB sampling [224, 226] or use of multicopy genes [230]. Assay performance may be different between AF and FB, with reported concordance between 73% and 99% [210,213]. Although

(transient) ZIKV was reported in AF and FB in fetuses of women with proven infection during pregnancy [21], there are no quantitative data on FB/AF in Zika available at this point [231].

POSTPARTUM DIAGNOSTIC TESTING (FIGURES 4A – 4D)

Postpartum sequelae of fetal infections have been observed for TOXO, RV, CMV and ZIKV. Although literature is not consistent on this issue, fetal anemia following PB19 infection may result in severe postpartum sequelae [232]. In general, timely postpartum diagnosis is hampered by low sensitivity of IgM testing [33,36,40,205,228,233,234,235], the presence of maternal antibodies, and the high proportion of asymptomatic CMV or TOXO infected newborns [31,128,224,228,236], unlike for RV [237]. As a consequence, ascertainment of congenital disease typically requires longer-term follow up, posing challenges to the differentiation with postpartum infection [40,238,239].

IgM positivity in cord blood or peripheral blood in newborns <24 hours in RV and CMV confirms prenatal infection when supported by viral load testing [233,240]. IgM assay performance is better when testing is done more selectively, in symptomatic neonates as reported for TOXO [218]. Contamination with maternal blood should be excluded within the first 10 days postpartum if first sample was taken from CB, as shown for TOXO[35].

The majority of IgG detected at birth will be from maternal origin [31,241], but may be neonatal [242]. Generally maternal IgG is assumed to persist for less than 6 months [243,244], but for example in TOXO persistence of IgG level at 12 months is used to confirm or exclude congenital toxoplasmosis and IgG immunoblot is used to overcome the uncertainty about IgG origin [205,245]. Use of immunoblot or other multi-antigen assays early postpartum [249] has shown to provide an opportunity to differentiate congenital from non-congenital infection by comparing maternal and neonatal antibody binding patterns [246,247].

The feasibility of using differences in AI for this purpose has also been studied, e.g. for TOXO and CMV [247,248,249,250]. Slow IgG AI maturation in neonates, in combination with IgM correlated with congenital RV [251,252], identical neonatal and maternal AI excluded congenital toxoplasmosis [35].

Molecular testing of neonatal blood or urine has generally good specificity [239,253] with higher viral load, and longer RNA/DNA persistence in symptomatic babies, particularly in urine or throat samples for selected pathogens within a selected time frame after birth. [239,254,255,256]. Viral load has been used to differentiate congenital from postpartum infection, when early samples are available, but it is not clear if these findings can be generalized [257].

EFFORTS AT IMPROVING DIAGNOSTIC ACCURACY

There have been many efforts to improve diagnostic accuracy, however, this has not yet resulted in significant improvements. These efforts include development of recombinant (multi-) proteins and peptide specific tests using different techniques (e.g., immunoproteomics) [258] in (multiplex) assays to improve sensitivity and specificity [143,259,260,261], distinction between primary and postprimary infection [262,263,264], timing [265], and transmitters [266]. For example, recombinant proteins in novel avidity assays reported a PPV of >85% [267] and were better suited for IgG detection in TOXO [268] or could serve as proxies for functional antibody measurements like virus neutralisation in RV [269]. Multiplex assays are used for simultaneous detection of different antibodies in TORCH [34,108,113,143] , which is important for differential diagnostic approaches. However, assays still have the performance limitations of the standard assays described. Microarray based assays are developed to improve simulatenous testing of antibodies of different pathogens, including (extended) TORCH [270]. Use of dry blood spots in multiplex

serological assays allows use of small volumes, and shorter diagnostic delay [271,272,273], with potential better assay performance on for example plasmonic gold chip multiplex immunoassay platforms as shown in TOXO [274,275,276]. Cell mediated immunity (CMI) assay data (IGRA, ELIspot) particularly come from CMV but is not routinely used. Higher CD4+/CD8 proliferative T-cell response was associated with primary infection [277,278,279,280,281], improving assay sensitivity of low IgG avidity [282], but also reporting different assay performances for example in primary infection and transmitters [283]. Since CMI in the neonate is never from maternal origin, it is hypothesized that it might aid in differentiating maternal from foetal ZIKV infection.

Rapid point of care testing, such as immunochromatography [98], loop-mediated isothermal amplification (LAMP) [141,284] or digital microfluidic (DMF) diagnostic platforms [285] studied for different pathogens, may further reduce time to first positive test, increase sensitivity and/or decentralised availability in resource limited settings. Such developments are also reported for ZIKV [286,287, 288,289] Alternative, novel methods in amniotic fluid samples include comparisons of metabolic profiles (metabolomics) of transmitter vs non-transmitter infections [290,291], cytokine profiles [292] or peptidome prognostic classifiers [229] to differentiate infected from non-infected fetuses, or distuinguish symptomatic from asymptomatic infections postpartum. Such developments are particulary important as they may provide early (prenatal) information on the risk of overt clinical congenital disease postpartum.. Other non-pathogen related methods are those comparing differential gene or protein expression between fetal cells and maternal cells [293]. In analogy with previously developed tests for non-infectious prenatal screening [294,295], genes associated with neurodevelopment were studied as biomarkers in cell-free RNA transcripts in AF samples [296]. Paper-based cell-free RNA was recently evaluated for rapid point of care testing of ZIKV [297].

CONCLUSIONS

Our review of approaches to diagnose acute maternal infection, determine vertical transmission risk and establish presence or absence of congenital infection has shown similarities but also large variation in approaches between pathogens, risking under-exploration of methods for optimal diagnostics. Present diagnosis of TORCH and ZIKV infections is primarily based on serological testing with a focus on IgM and/or IgG detection, for which a variety of commercial assays is available. These assays show variable performance and may not differentiate between primary and non-primary infections [115], persistence or may be limited by cross reactivity [108,112]. A positive serological test thus always requires confirmatory testing, including IgG avidity index determinations, immunoblots, virus neutralisation and molecular testing [43,212,224].

Use of different assays and lack of (international) standardization hamper the interpretation of and agreement between different studies [298], despite availability of (WHO) recommended antigens, primers and probes [299]. Efforts to improve detection of primary infection and timing in pregnancy have not yet resulted in reliable biomarkers for fetal or congenital disease risk [300]. Even if protocols and/or algorithms are in place, variability between assays interferes with unambiguous and timely decision making [301].

Thus predefined (more) generic approaches with standardized diagnostic assays and algorithms are needed to improve adequate and timely diagnosis of (primary) maternal infections, and subsequent postpartum congenital disease [302], particularly in low endemic settings where suboptimal diagnostic performance may have an increased risk of false positive outcomes. Lessons to draw from this review for novel challenges such as ZIKV are to directly combine methods [52], increase epitope specificity (e.g., avidity, immunoblot, virus neutralisation) and implement paired mother-fetus and/or mother-child testing, as was

445 recently reported for ZIKV neutralising antibodies [303]. Differences in background exposure
446 to ZIKV and other flaviviruses will have an (age-dependent) effect on cross-reactivity and
447 interpretation of protein-driven assays, such as IB or micro-array analysis. In these instances,
448 CMI might be explored as alternative method to differentiate maternal from congenital ZIKV
449 infection. Standardisation of (validated reference) methods is critical in order to compare
450 different methods and might need (a) reference centre(s) to confirm acute infection. There is a
451 plethora of studies describing potentially improved diagnostics for the TORCH complex
452 infections, including ZIKV. Exploration of the broad range of published methods is important
453 to improve diagnostic algorithms. In the meantime, it is essential to raise awareness among
454 medical microbiologists and treating physicians about the limitations of the presently applied
455 tests and algorithms, guiding protocol development for diagnostic testing of (novel) infections
456 such as ZIKV and optimise diagnostic algorithms, for the different geographic and resource
457 settings. Given the observed disconnect between the different pathogen specialist fields, we
458 conclude that there is a clear case to be made for an integrated TORCHeZ diagnostic
459 challenge.
460

FIGURE LEGENDS

Figure 1:

Seroprevalence, maternal infection risk, fetal transmission risk and congential infection risk of the following selected infections: TOXO, PB19, Rubella, CMV and ZIKV.

Legend:

* seroprevalence at childbearing age

** IR/yr= maternal annual infection rate

$ FTR= fetal transmission rate

CIR = congenital infection rate= number of congenital infections per 1000 live births (TOXO) or per 1000 pregnancies (RV, CMV, PB19, ZIKV)

Figure 2: Routine maternal diagnostic methods: sensitivity and specificity, PPV, NVP median point estimate +/- 95% CI

Legend:

Abbreviations: PPV: positive predictive value, NPV: negative predictive value, 95% CI: 95% confidence interval

References figure 2:

TOXO (n=28): 48,99,113,134,143,150,153,160,161,162,163,165,170,171,175,177,179, 183,189,195,199,200,201,204,205,209,262,304

PB19 (n=14):29,36,53,79,84, 86,121,146,176,197,211,305,306,307

RV (n=9):59,108,137,149,151,186,204,304,308

CMV (n=24):23,53,92,101,112,128,145,148,155,156,159,166,169,173,178,190,196, 204, ,206,250,265,281, 304,309

ZIKV (n=15):289,310,311,312,313,314,315,184,316,317,318,319,320,321,322

Figure 3: Routine fetal diagnostics methods: sensitivity and specificity, PPV, NVP median point estimate +/- 95% CI

Legend:

Abbreviations: PPV: positive predictive value, NPV: negative predictive value, 95% CI: 95% confidence interval

References figure 3:

TOXO (n=13): 33,43,126,154,164,209,212,226,227,228,230,323,324

PB19 (n=5): 79,213,220,222,223

RV (n=1): 323

CMV (n=10): 89,129,169,210,222,223,224,229,277,323

Figure 4: Routine neonatal screening methods: sensitivity and specificity, PPV, NVP median point estimate +/- 95% CI

Legend:

Abbreviations: PPV: positive predictive value, NPV: negative predictive value, 95% CI: 95% confidence interval

References figure 4:

TOXO (n=9): 174,201,212,228,234,246,248,249,325

PB19 (n=2): 36,85

RV (n=2): 233, 252

CMV (n=11): 102, 115,128,158,159,215,238,250,253,277,326

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FIGURES